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THE NATURE OF GLIOMAS AS REVEALED BY ANIMAL EXPERIMENTATION *

H. M. ZIMMERMAN, M.D.

From the Laboratory Division, Montefiore Hospital, New York 67, N.Y.

Primary intracranial neoplasms in man comprise from 2 to 5 per cent of all tumors in the body. Of these neoplasms, somewhat less than half belong to the glioma group. The latter comprises at least seven universally recognized, distinctive types as well as a number of related subtypes. The major types are: astrocytoma, astroblastoma, ependymoma, glioblastoma multiforme, medulloblastoma, oligodendroglioma, and spongioblastoma polare. To the subtypes belong, among others, the ependymoblastomas, ganglioneuromas, and medullo-epitheliomas.

The problem of identification and classification of tumors of the glioma variety has not been simple, mainly for two reasons. One is that neurosurgical pathology is still in that early developmental stage which is preoccupied with descriptive morphology and with finding new tumor types to classify. Thus it is still considered somewhat of an achievement to have divided the astrocytoma into the piloid, fibrillary, and protoplasmic types, even though the histogenesis and biologic behavior of this tumor does not seem to warrant such subclassification. The other is that a vastly complicated terminology has developed which has greatly discouraged students in this field. The concept is also current that a battery of complicated and difficult staining techniques is essential for the identification of the various gliomas. The general pathologist has shunned the field of neurosurgical pathology because of his belief in the almost insurmountable complexity of the intracranial neoplasms.

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Terminology of Gliomas

Gliomas have their origin from the cells of the glial stroma of the brain and spinal cord. These cells include the astrocytes, oligodendroglia, and ependymal cells. The fourth basic glial cellular component, the microglia, evidently does not participate in the production of neoplasms; at least there are no generally recognized tumors which can be traced to this cell type.

The morphologic variations in gliogenous neoplasms with the attendant complexity in their classification was brought into some order by Bailey and Cushing[1] in 1926. Their nomenclature of gliomas was based on the histogenesis of the nervous system. The classification they advocated has the advantage of long usage and wide acceptance. It has the added advantage of specific terminology for morphologically distinct entities and permits fairly accurate prognosis. It is their classification of the seven common types of glioma which was given at the outset of this presentation.

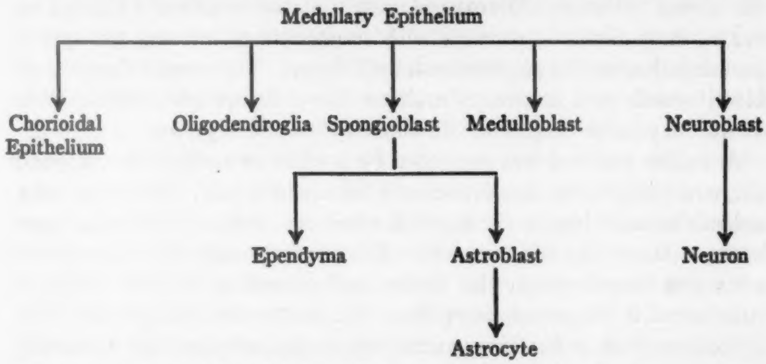
The obvious disadvantages of this classification are the rather large number of individual tumors included and the fact that certain gliomas are derivatives of more than one basic glial type; that is, certain gliomas are of "mixed" origin and defy classification. Efforts have been made from time to time to simplify the terminology, and recently by Kernohan, Mabon, Svien, and Adson.[2] These workers suggested a classification which is based on the idea that gliomas arise from the pre-existing adult astrocyte, oligodendrocyte, and ependymal cell. This concept provides three basic glial neoplasms: astrocytoma, oligodendroglioma, and ependymoma. Kernohan and his co-workers assumed that more malignant neoplasms develop from these basic types by a process of dedifferentiation. For example, in their classification the glioblastoma multiforme is merely an astrocytoma of grade III or IV as to degree of malignancy.

There are at least four important faults to be found with this approach. One is that in reality the classification is no simpler than the one adopted by Bailey and Cushing.[1] If four numerical designations for each of the three basic types of glioma are employed, as proposed by Kernohan and his associates,[2] to designate morphologically distinct tumors, there are still twelve gliogenous neoplasms to be diagnosed. Another objection is the one inherent in all classifications which resort to numerical grading of degrees of malignancy. It implies a prophetic capacity on the part of the pathologist which is often not fulfilled by the clinical outcome. Still another objection is the failure of the classification to provide for the medulloblastoma in a scheme of tumors originating from the three adult glial cell types. But perhaps the major

fault with the scheme is the assumption that each glioma takes origin from a single cell, such as an astrocyte, and that the developing tumor is merely the result of proliferative activity on the part of that glial element. Actually, as will be shown presently, many if not all gliomas contain mixtures of different proliferating gliocytes. Experience has further indicated that classification of any one tumor is valid only after extensive sampling, a fact which was stressed by Scherer[3] in 1940.

The Nature of Human Gliomas

It is generally accepted that the different glial cells of the mature nervous system, with the possible single exception of the microglia, are all derived from embryonal neurectoderm as shown in the schema.



Schema to show origin of adult cellular constituents of the brain from medullary epithelium.

This origin the glia share in common with the neurocytes. Further, the primitive neurectoderm or medullary epithelium differentiates by recognizable stages to the fully mature glia. These adult cells may give rise, under certain conditions, to glial neoplasms by dedifferentiation. Implicit in this concept are two conditions: (1) that the adult glial cells undergo malignant change and by proliferation give rise to the neoplasm; (2) that the proliferating cells proceed through stages of dedifferentiation ultimately to achieve degrees of uniformity which permit classification.

Almost every glioma contains, in addition to the distinctive cells from which its name is derived, variable numbers of other glial elements. Classification of a gliogenous neoplasm is thus based upon the identification of the most malignant cells present and/or the predominance of one kind of cell. Yet every neuropathologist frequently finds himself in a dilemma when he abides strictly by these criteria for classification. An example of this problem in tumor diagnosis is offered by

the following case of a white male, 53 years old, whose frontal lobe biopsy (Fig. 1) at an exploratory craniotomy revealed a cystic astrocytoma. Nothing in this neoplasm hinted of the course this patient was to follow, for within 1 year he returned for re-operation because of recurrence of the tumor. The second operation yielded an astrocytoma once again (Fig. 2). This time the tumor was no longer cystic and it was considerably more cellular, but the presence of calcium salt deposits was in keeping with a diagnosis of a benign astrocytoma. Yet within 7 months the patient was back in the hospital and after a stormy course of less than 4 months he succumbed. At necropsy a huge tumor was found replacing much of the right cerebral hemisphere, infiltrating the basal ganglions, and extending into the left hemisphere by way of the corpus callosum. Microscopic study of the neoplasm (Fig. 3) revealed large zones of necrosis with spongioblasts forming the typical pseudopalisades of a glioblastoma multiforme. There were thrombosed blood vessels and numerous multinucleated tumor giant cells. Parts of the very large neoplasm still consisted of astrocytoma.

A similar problem was presented by a white sales engineer, 31 years old, who had gradual loss of memory for about 1 year. Increasing somnolence brought him to the hospital where air studies disclosed a tumor in the region of the third ventricle. This was explored and a firm tumor mass was found within the cavity and extending into the adjacent structures. A biopsy specimen from the intraventricular portion (Fig. 4) consisted of a fibrillary astrocytoma. Experience with unusually large, hyperchromatic cells in an otherwise benign appearing astrocytoma counseled a cautious diagnosis, but re-examination of the tissue revealed only the benign astrocytic tumor. The patient failed to respond to the subtotal removal of the neoplasm and died in less than 1 week after craniotomy. The remainder of the tumor in the paraventricular region (Fig. 5) disclosed cellular pleomorphism, giant tumor cells, numerous mitotic figures, perivascular collars of lymphocytes, and necrotizing angiitis. All of these features are common to glioblastoma multiforme.

Neither of these patients received any therapy for their tumors except surgery. The next patient differed in this regard. He was a young man who had a tumor removed by Dr. Leo M. Davidoff in September, 1949. This proved to involve the brain stem, more especially the medulla oblongata, and some of it extended out of the medulla sufficiently to permit a biopsy (Fig. 6). The tumor was an ependymoma with typical cart-wheel perivascular rosettes. The patient made a surprisingly good recovery from his operation and radiation therapy, and was able to return to work for a period of about 2 years. He then

began to slip gradually and finally died in June, 1953. Remnants of the original tumor could still be made out at necropsy in and around the medulla, but much of the neoplasm was now composed of strikingly pleomorphic cells, giant tumor cells, and spongioblasts (Fig. 7). This tumor was no longer, if indeed it ever was, a "pure" or uniform ependymoma. It could best be classified as a malignant glioma if not a glioblastoma multiforme.

The final illustrative case is that of a white male, 30 years of age, who developed increasingly severe headaches for a full year preceding hospitalization and exploratory craniotomy. A large right frontoparietal tumor was found and excised. Microscopically, this tumor (Fig. 8) proved to be a cystic astrocytoma of strikingly uniform appearance. After operation, the patient never felt completely well, experiencing some mild headaches at first which gradually increased in severity. He stated that he felt his "skull just bouncing up and down." He developed photophobia and nausea. A course of x-irradiation totaling 6200 r. was given over the right hemisphere but without noticeable improvement. The patient's condition deteriorated and he died 4 years after operation. The tumor, which had recurred, was found to occupy much of the right cerebral hemisphere at post-mortem examination. Parts of it still presented the appearance of the cystic astrocytoma seen at biopsy; parts were composed of large protoplasmic astrocytes (Fig. 9) with coarse unipolar and multipolar processes; and parts had the wildly pleomorphic appearance of a glioblastoma multiforme (Fig. 10), with zones of necrosis and hemorrhage and a profusion of spongioblasts, astrocytes, and other glial cells.

To these examples of gliomas which are "mixed" tumors at the start and those which apparently become transformed from one type to another spontaneously, or following operative intervention or radiotherapy, may be added many more. Indeed, it is the exception rather than the rule to find gliomas which are composed of but a single cell type. Added examples can be given of oligodendrogliomas which are in part ependymomas, or astrocytomas, or even glioblastoma multiforme, and of polar spongioblastomas which are partially glioblastomas.

The Nature of Experimental Gliomas

At this point we may perhaps examine with profit the contributions which animal experimentation has made to an understanding of the complexities which surround the subject of human gliomas.

For nearly 15 years my associates and I have produced gliogenous neoplasms in several different inbred strains of mice by the expedient of implanting minute pellets of chemical carcinogens in different loca-

tions within the brains of these animals.[4-7] In this way it has been shown that many different adult and morphologically distinct glial cells begin to proliferate almost simultaneously under chemical stimulation to produce a neoplasm. This principle is illustrated in the photomicrograph (Fig. 11) of an incipient glioma which was produced by inserting a pellet of methylcholanthrene into the lateral ventricle of a mouse. The early lesion already shows some injury to, and perhaps also some proliferation of, the ependyma. But in addition there is a lively subependymal glial response in which a number of cells participate. More clearly perhaps can be seen the simultaneous proliferative activity of adjacent glial cells in another animal (Fig. 12). Here the yellow pigment of the carcinogen is found in several altered cells from which the glioma derives. Since the tumor springs into being, so to speak, from several progenitors, it is almost never a "pure" tumor, that is, composed of one cell type, but rather a mixture of several different cell types.

More than 12 years ago Dr. Arnold and I described a mouse which developed a "mixed" or multiple glioma (Fig. 13) in response to methylcholanthrene.[4] Part of the neoplasm was composed of spongioblasts forming pseudopalisades on the edges of necrotic foci as seen in glioblastoma multiforme and part was composed of an oligodendroglioma. More recently, Dr. Maier and I, recognizing that very few experimental gliomas were "pure" tumors and that most were constituted of several different cell types, that is, that they were "mixed," performed the following transplantation experiment.[8] Portions of a single primary brain tumor were implanted subcutaneously into a series of homologous animals. In this way it proved possible to establish sublines of two or more different "pure" tumors. From a single primary malignant glioma, one subline of a "pure" oligodendroglioma (Fig. 14) was obtained which has remained morphologically constant in over 100 serial transplants, and from the same primary tumor another subline of a "pure" ependymoma (Fig. 15) has been derived which has also remained constant throughout a similar number of transplants. This experimental evidence suggests that the glioma is multipotential, by which is meant that it has the ability to become one or another of the seven major types of this class of tumor. The preponderance of neoplastic cells of one type is the sole justification for the appellation chosen for any one tumor as a whole.

Certain experiments performed by my associate Alfred Cohn[9] have thrown some light on the reason for the preponderance of one cell type in a glioma which is "mixed" and therefore contains cells of quite other

types. Starting with a mouse glioma which had the appearance of an ependymoma (Fig. 16) in the original tumor and in a long series of subcutaneous transplants, he grew fragments of the rodent neoplasm in the allantoic membrane of the chick egg with rather startling results (Fig. 17). Each egg passage produced an amorphous mass of tumor cells whose identity even as gliocytes was questionable. When, however, these cells were transferred from the egg to the mouse, the characteristic morphology of an ependymoma was reconstituted. In as many as eight consecutive egg passages the tumor remained amorphous, only once more to become an ependymoma when transplanted subcutaneously to a mouse. These experiments emphasize the important influence which the environment of a tumor has on its microscopic structure. It is conceivable that environments or host factors exert important effects on the ultimate appearance which gliomas assume even in man. Heretofore, emphasis has been directed entirely to the tumor cell as the determining factor in tumor appearance.

Another factor which in large measure determines the appearance or type of an experimental glioma is the location in which the chemical carcinogen is placed within the brain. Dr. Maier and I[8] showed that when the chemical is within the ventricular system, an ependymoma or an ependymoblastoma resulted; in the subcortical white matter, a glioblastoma multiforme developed usually, and, far less frequently, an astrocytoma; in the occipital lobes, the usual tumor was an oligodendroglioma; in the corpus callosum, a spongioblastoma polare; in the cerebellum, the induced tumor was most often the medulloblastoma. This topographic distribution of the experimental gliomas corresponds closely to that of the similar tumors which occur spontaneously in man.

There are other factors which influence the microscopic appearance of gliogenous neoplasms. In experiments dealing with the effect of single doses of x-irradiation on the rate of growth and microscopic appearance of an ependymoma, my associates Netsky, Freid, and Corsentino[10] showed that microscopically detectible changes were obtained with doses as low as 400 r. Subcutaneous transplants of the tumor in control animals showed little or no necrosis and the tumor cells were arranged in the classical rosettes of an ependymoma (Fig. 18). Within 3 days after irradiation of the tumor with a single dose of 400 r. there was partial loss of rosette formation and a concomitant appearance of bizarre tumor cells (Fig. 19). The tumor so treated grew more rapidly than the controls.

With a dosage of 1200 r. to the subcutaneously transplanted ependymoma there was a definite diminution in tumor size at first, but then

its rate of growth exceeded that in the control animals. At 13 days after irradiation the neoplasm (Fig. 20) contained some cells which could be identified as ependymoma but, in addition, there were present many huge multinucleated tumor giant cells and a dense stroma of glial fibers.

The largest single dose employed was 5000 r. Within 4 days after treatment the tumor (Fig. 21) had lost the appearance of an ependymoma. Rosettes were absent and the individual cells were swollen and had pale nuclei. Small foci of necrosis appeared. By the 9th day following treatment the tumor was shrunken and barely palpable, and persisted thus until the 20th day. A photomicrograph (Fig. 22) of the tumor on the 14th day after treatment showed no cells which could be recognized as of ependymal origin. The viable cellular elements consisted of huge, bizarre, and often multinucleated structures. The tumor resumed growth by the 20th day and thereafter increased in size more rapidly than in the controls. Microscopically (Fig. 23), 20 days after the x-ray dosage of 5000 r. the neoplasm still had but scant resemblance to the original ependymoma, but some ependymal cells were in evidence. The tumor thereafter rapidly regained its normal appearance and by the 25th day it was indistinguishable from the control tumors.

These examples will suffice to illustrate the point that gliomas are not always what they seem. The environment in which they find themselves, their sites of origin, and such external influences as roentgen irradiation, all affect their appearance. Perhaps there are many more equally important and as yet undiscovered influences.

Summary and Conclusions

Animal experimentation with gliomas produced with chemical carcinogens has contributed to the clarification of a number of the problems which surround human neoplasms of this type. It has shown that under chemical stimulation many different adult and morphologically distinct glial cells begin to proliferate almost simultaneously to produce a glioma. The resulting tumor is therefore almost never a "pure" glioma composed of cells of one type, but rather a mixture of cells of several different types. In a true sense it is a multipotential neoplasm; i.e., it has the cellular composition and ability to become one or another of the seven major types of this class of tumor. Transplantation experiments have actually proved the validity of this concept, for by judicious selection of explants from a "mixed" glioma, multiple "pure" gliomas may be created by transplantation into homologous animal species. This experience justifies the view that all experimental gliomas are really variants of one tumor type, in the same sense that Hodgkin's

granuloma, lymphosarcoma, and reticulum cell sarcoma are variants of malignant lymphoma.

The principle of multipotentiality is equally applicable to the human gliomas. They, too, like the experimental tumors, are often composed of many different cell types. Some of them, like glioblastoma multiforme, also occasionally have a multicentric origin in different parts of the same or even in the opposite cerebral hemisphere. In these ways also the human gliomas are analogous to the malignant lymphomas. This raises an all-important question; namely, is there any justification for subclassifying the human gliomas?

There is a twofold answer to this question. Firstly, in the evolution of any given glioma a characteristic microscopic structure is usually attained which permits subclassification. The characteristic picture is provided by the predominating glial cell type, but may be influenced also by vascular proliferation, thrombosis, hemorrhage, and necrosis, and sometimes by the appearance of the most malignant of the glial cells participating in the neoplastic process. The second answer to the question, and the more important, is that clinical experience in many instances has shown the validity of prognosis based on the accepted schemes of classification.

What determines the sites of predilection of certain gliomas in the brain? A partial answer to this question is provided by animal experimentation as well as by study of human tumors. Repeated experiments have shown that ependymomas occur when carcinogenic agents are placed in contact with the ventricular wall; medulloblastomas originate almost exclusively in the cerebellum; oligodendrogliomas arise in the subcortical white matter of the cerebral hemispheres. The predominance and availability to malignant change of certain glial cells in different parts of the brain evidently determine the predilection of certain gliomas in different sites.

Animal experimentation has shown also that the environment or the host of a glioma is an important determinant of the type of tumor which develops. An example of this influence is the mouse ependymoma which grows as an undifferentiated malignant glioma in the chick embryo and reverts again to the ependymoma on transplantation to the mouse. And finally, animal experimentation with gliomas has shown that such external factors as x-irradiation may influence both rate of tumor growth and morphologic appearance.

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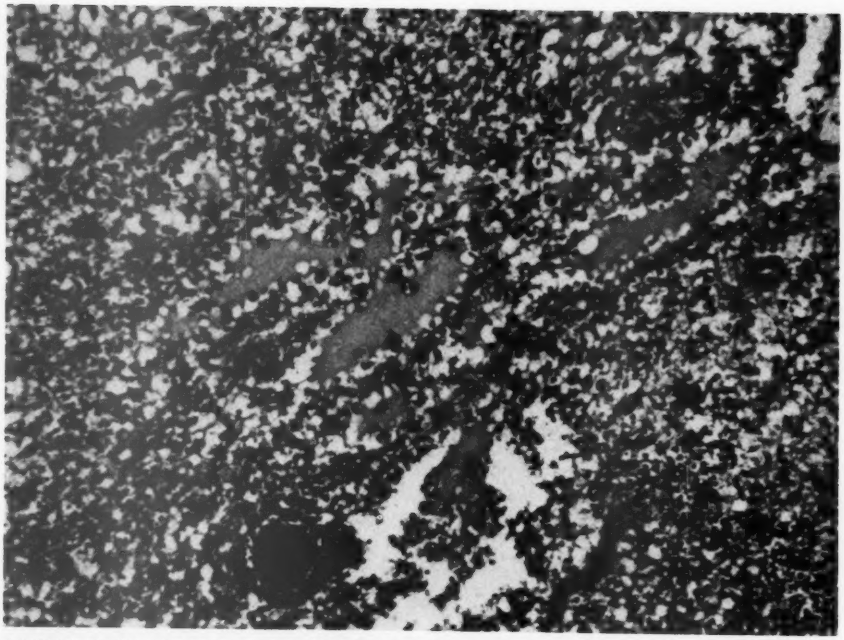
LEGENDS FOR FIGURES

FIG. 1. Cystic astrocytoma in biopsy material from frontal lobe at first operation upon a 53-year-old male. Hematoxylin and eosin stain. × 150.

FIG. 2. Astrocytoma with calcium salt deposits in biopsy material of frontal lobe tumor at second operation upon patient described under Figure 1. Hematoxylin and eosin stain. × 150.



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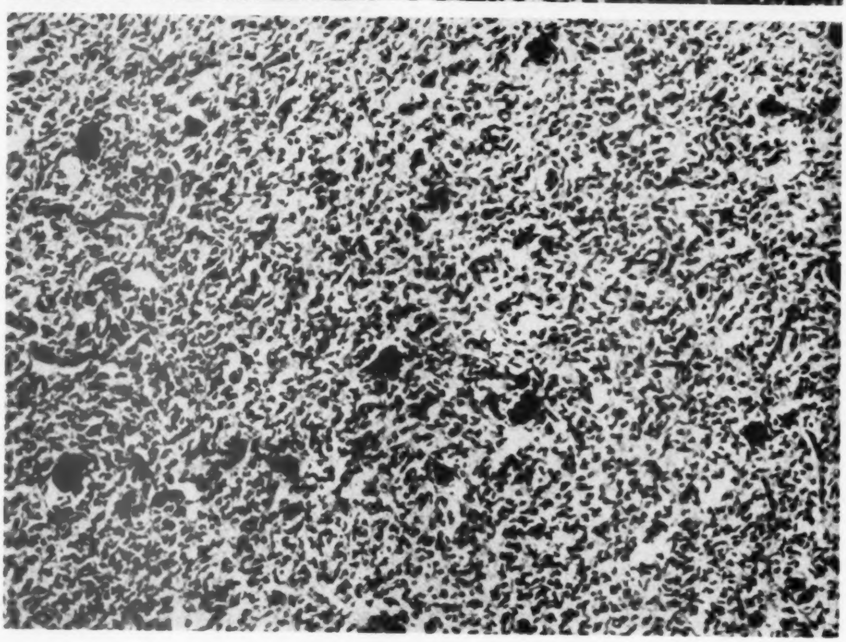

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FIG. 3. Glioblastoma multiforme found at necropsy in much of right cerebral hemisphere of patient described under Figures 1 and 2. Pseudopalisading of spongioblasts around foci of necrosis may be noted. Hematoxylin and eosin stain. × 150.

FIG. 4. Fibrillary astrocytoma in 3rd ventricle of a 31-year-old male. Hematoxylin and eosin stain. × 150.

FIG. 5. Necrotizing angiitis and multinucleated tumor giant cells in glioblastoma multiforme of paraventricular extension of the tumor in patient described under Figure 4. Hematoxylin and eosin stain. × 150.

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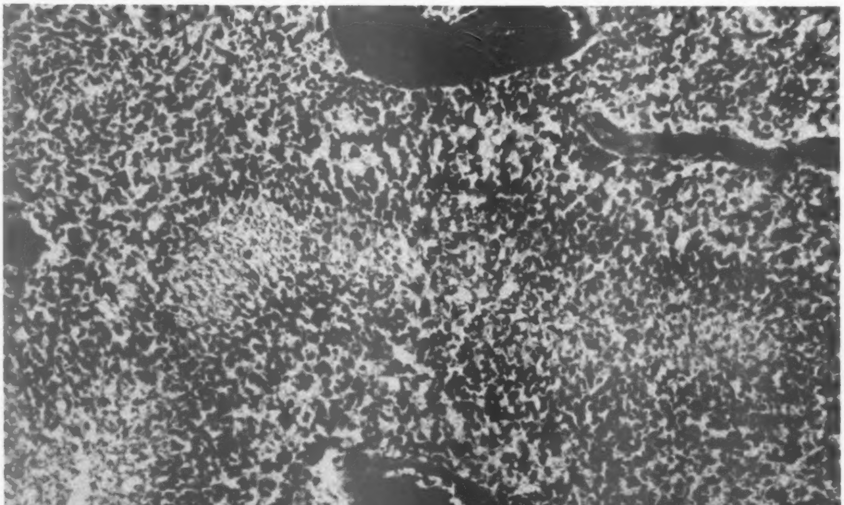

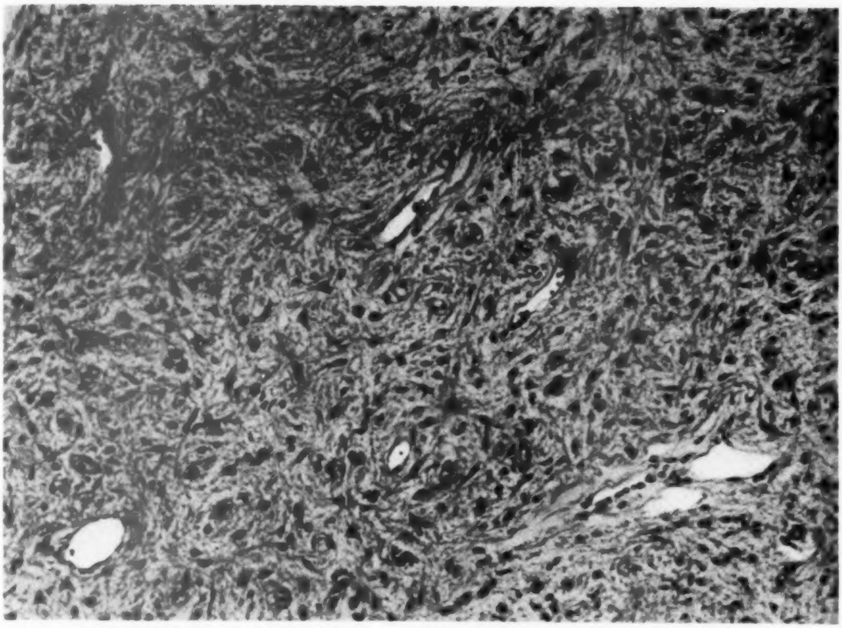

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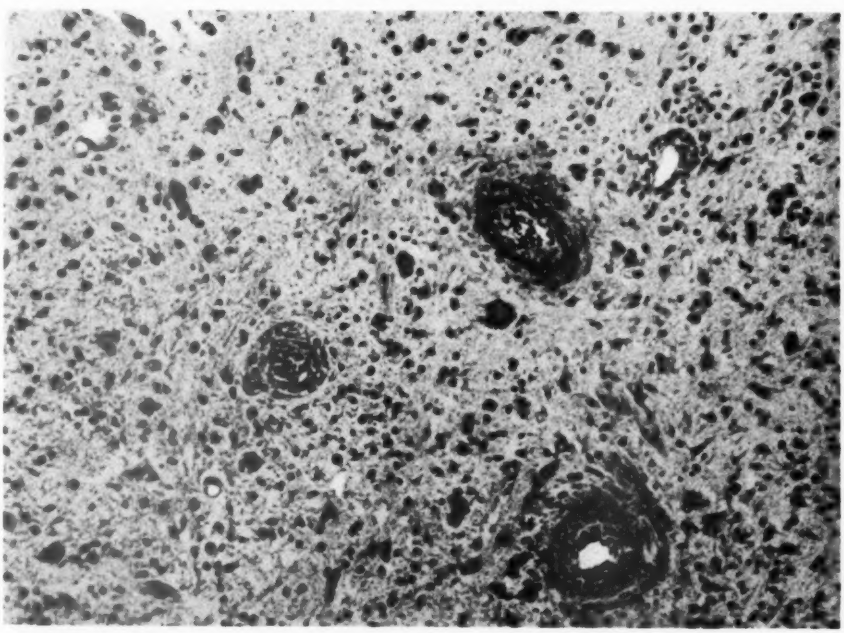

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FIG. 6. Ependymoma in medulla oblongata of a young male. Of note are the typical rosettes. Hematoxylin and eosin stain. × 150.

FIG. 7. Giant tumor cells and spongioblasts of the malignant glioma found at necropsy in patient described under Figure 6. Following operation and radiotherapy the patient made a good recovery for about 2 years. Hematoxylin and eosin stain. × 550.

FIG. 8. Cystic astrocytoma found at exploratory craniotomy in a 30-year-old male. Hematoxylin and eosin stain. × 150.

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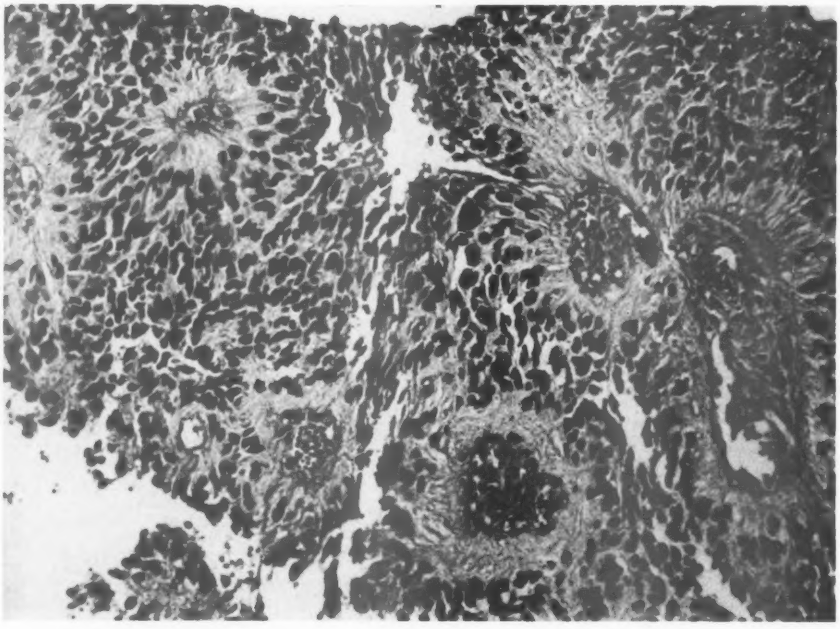

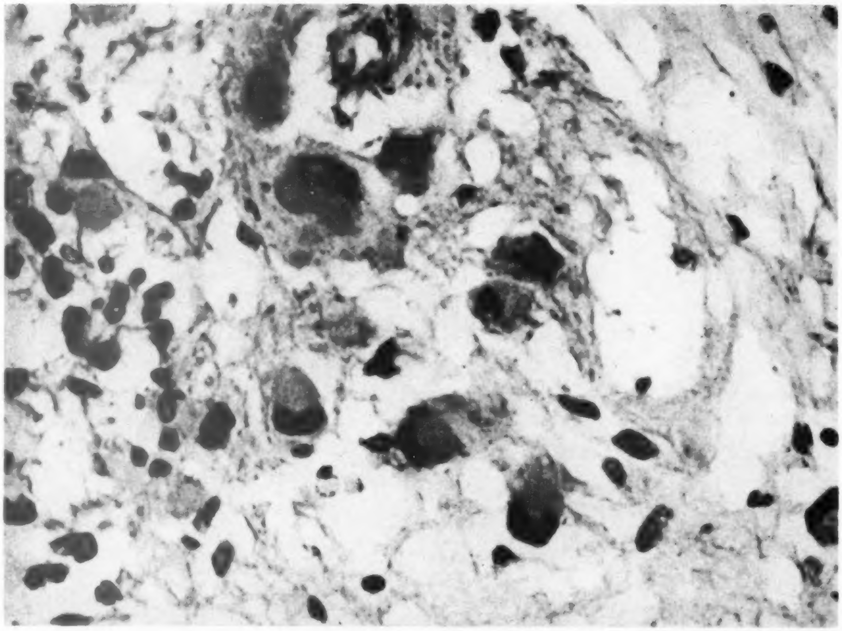

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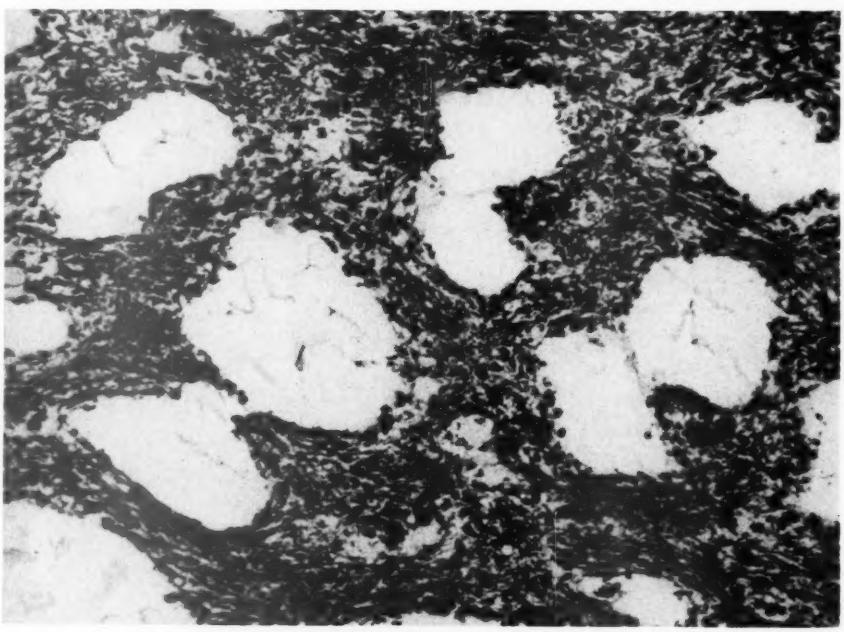

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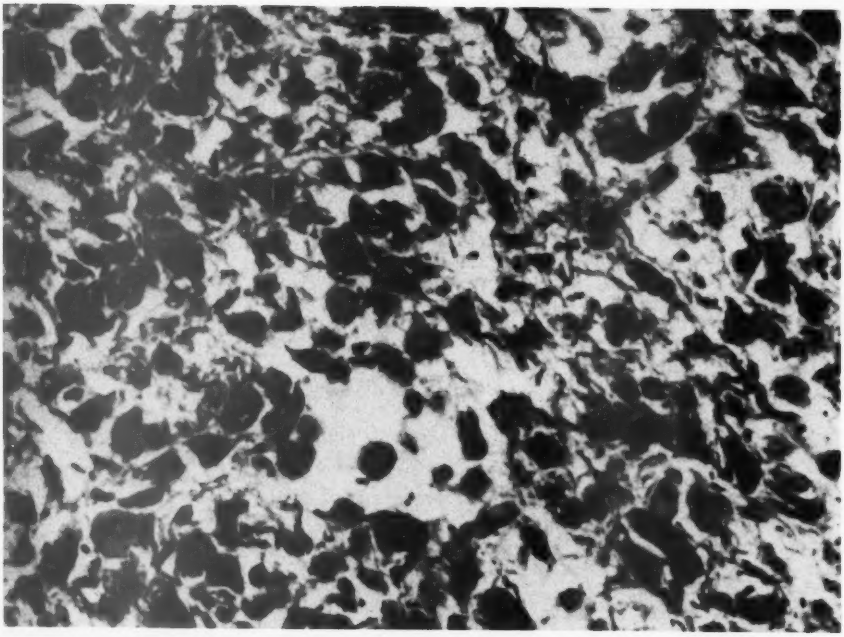
FIG. 9. Protoplasmic astrocytes in parts of tumor found 4 years later in right cerebral hemisphere of patient described under Figure 8. Hematoxylin and eosin stain. × 550.

FIG. 10. Pleomorphic appearance of glioblastoma multiforme in parts of same tumor shown in Figure 9. Hematoxylin and eosin stain. × 150.



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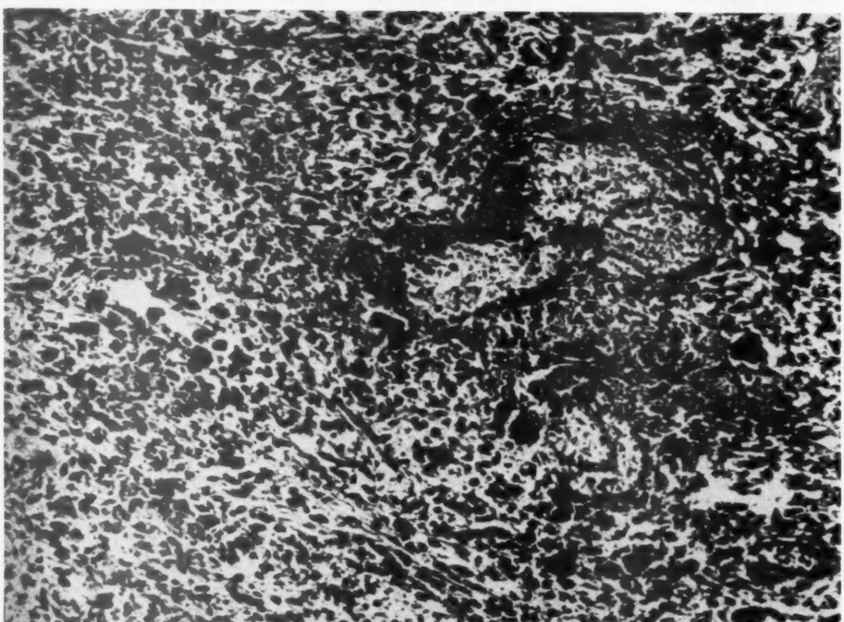

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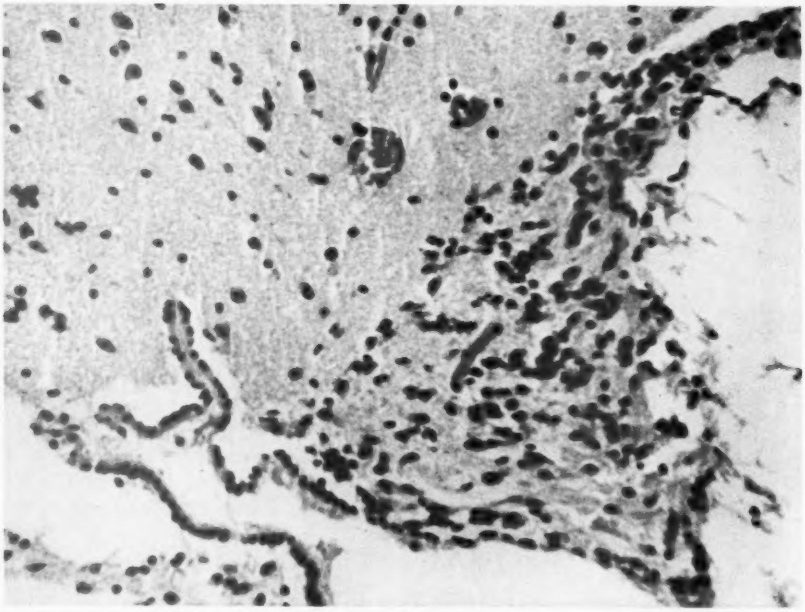
FIG. 11. Incipient glioma arising from ependyma and subependymal glia at site of methylcholanthrene implant. Hematoxylin and eosin stain. × 250.

FIG. 12. Incipient glial proliferation involving many different cell types simultaneously. Pigment of methylcholanthrene may be noted in several adjacent and altered gliocytes. Hematoxylin and eosin stain. × 750.



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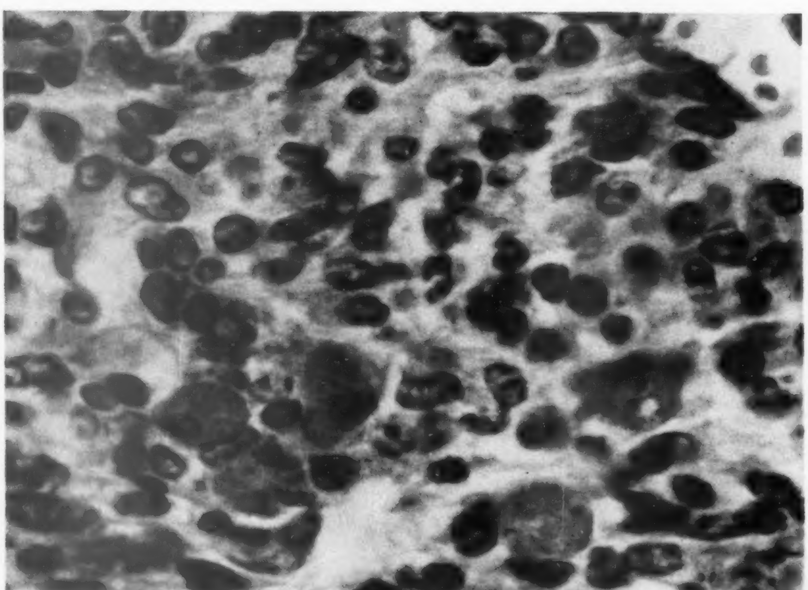

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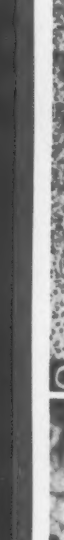
FIG. 13. Multiple or "mixed" glioma in mouse brain at site of chemical carcinogen. A. Drawing of tumor in right cerebral hemisphere. B. Destruction of basal ganglions by tumor. Hematoxylin and eosin stain. × 5. C. Spongioblasts forming a pseudopalisade as frequently seen in glioblastoma multiforme. Hematoxylin and eosin stain. × 100. D. Spongioblasts under higher magnification. Hematoxylin and eosin stain. × 400. E. Oligodendroglioma as part of the main tumor. Hematoxylin and eosin stain. × 400. Reproduced with permission from Cancer Research, 1941, 1, 919–938, Figure 7.



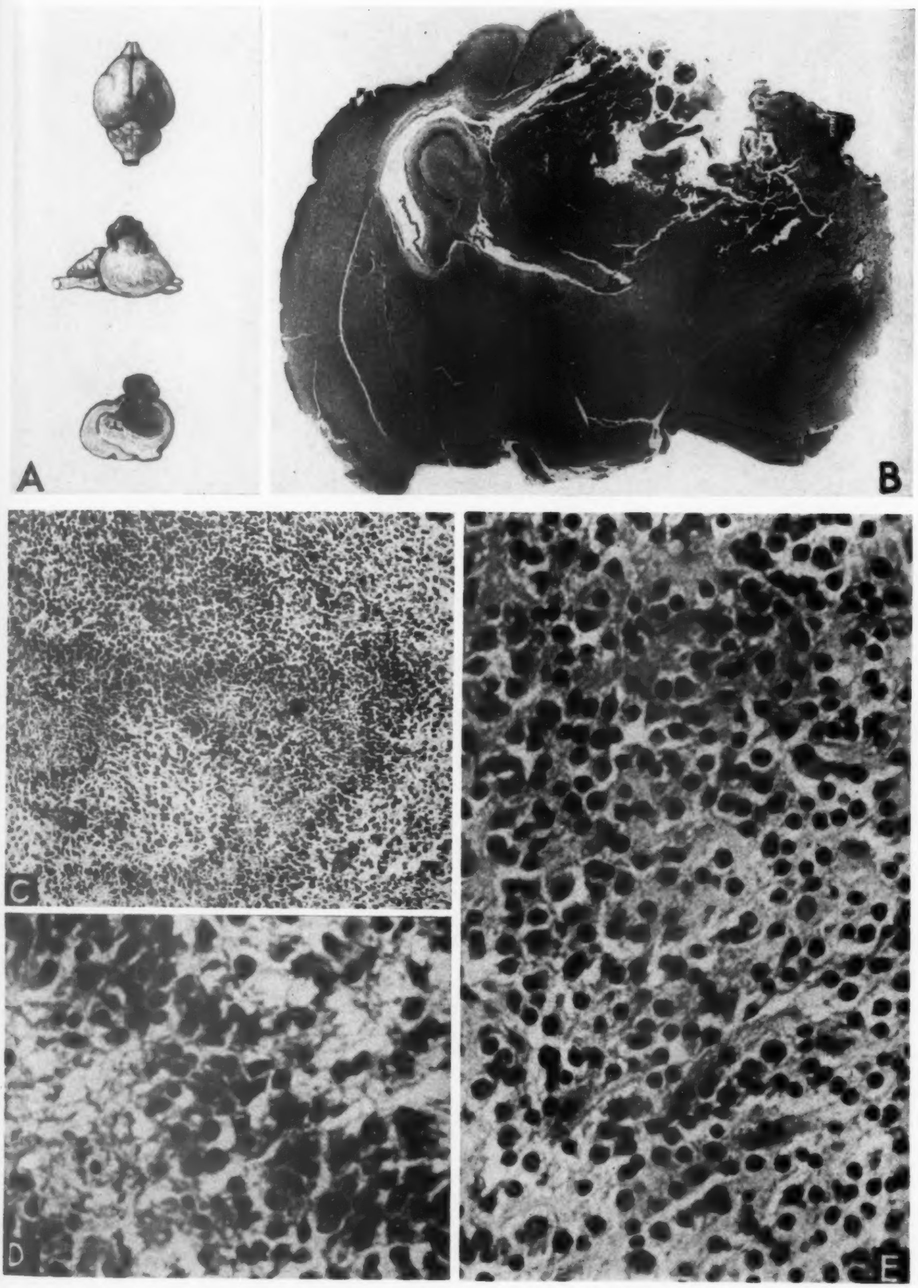


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FIG. 14. "Pure" subline of oligodendroglioma established by subcutaneous transplantation of a portion of a primary malignant glioma in a mouse. Hematoxylin and eosin stain. × 550.

FIG. 15. Ependymoma established as a "pure" subline from same primary "mixed" tumor described under Figure 14. Hematoxylin and eosin stain. × 550.

FIG. 16. Appearance of ependymoma produced in mouse brain with methylcholanthrene. Hematoxylin and eosin stain. × 185.

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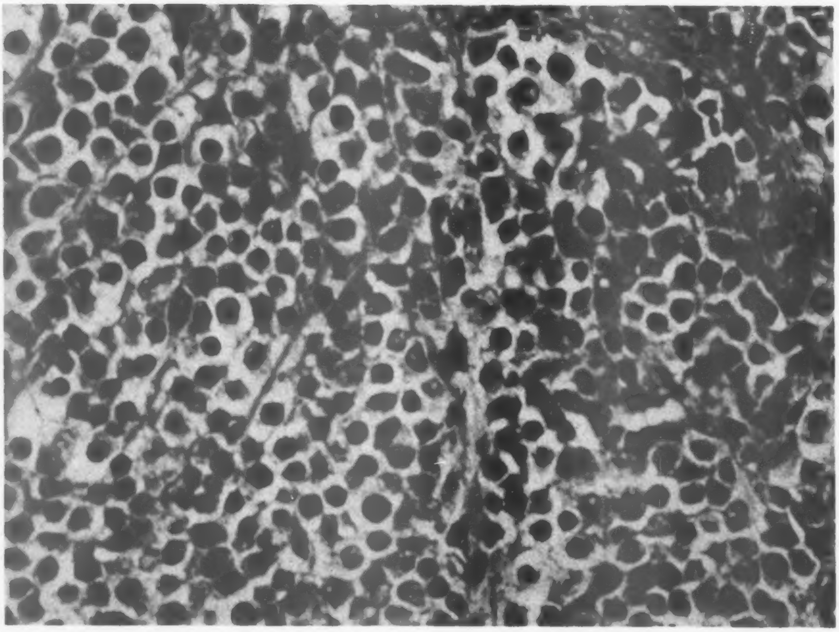

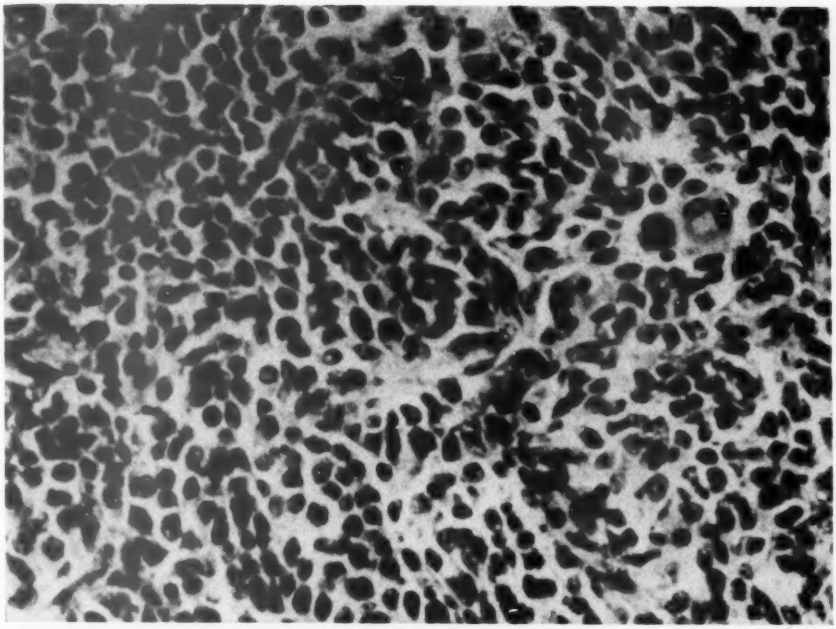

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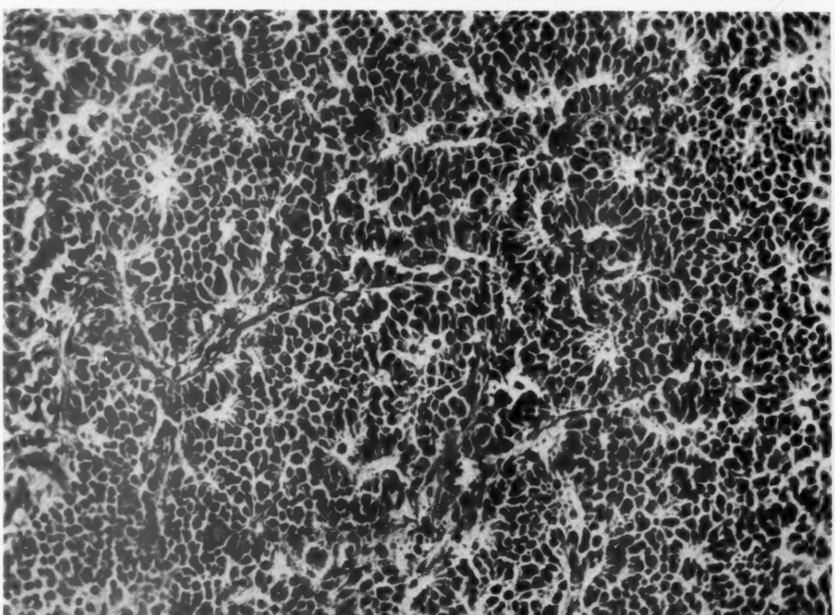

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FIG. 17. Appearance of glial tumor cells, originally a typical ependymoma in a mouse, growing in the allantois of the chick. Hematoxylin and eosin stain. × 550.

FIG. 18. Appearance of original ependymoma grown subcutaneously in the mouse. Control animal. Hematoxylin and eosin stain. × 185.

FIG. 19. Ependymoma on third day after x-irradiation with single dose of 400 r., showing partial loss of rosette formation and appearance of bizarre cells. Hematoxylin and eosin stain. × 185.

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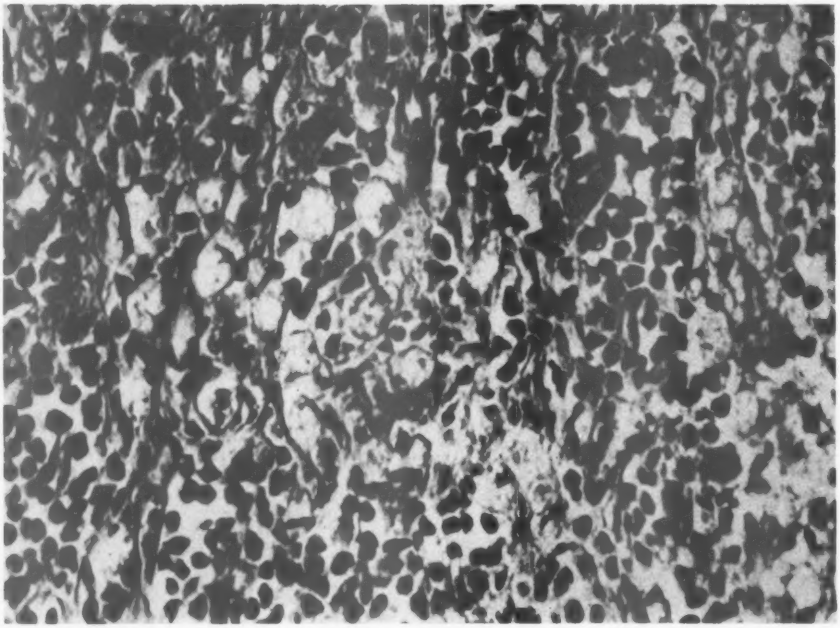

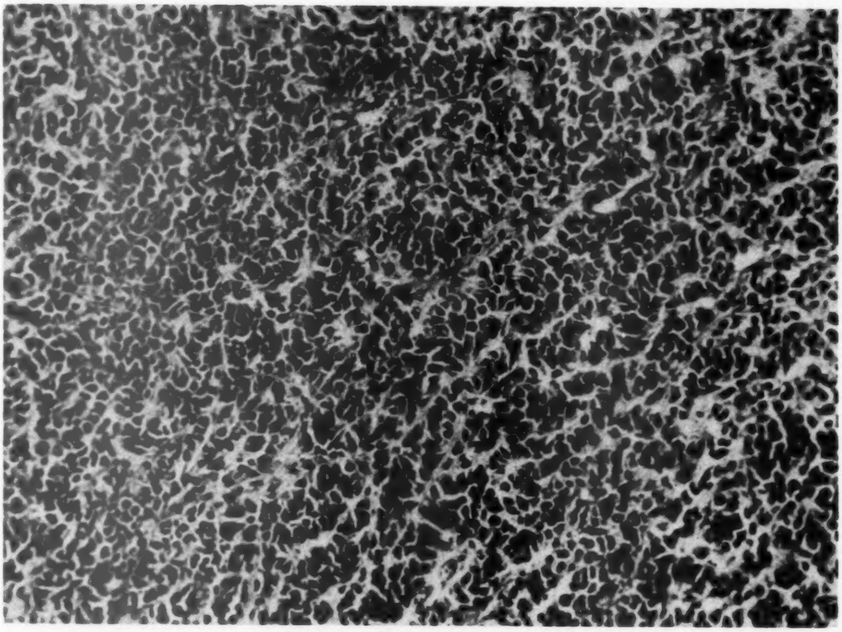

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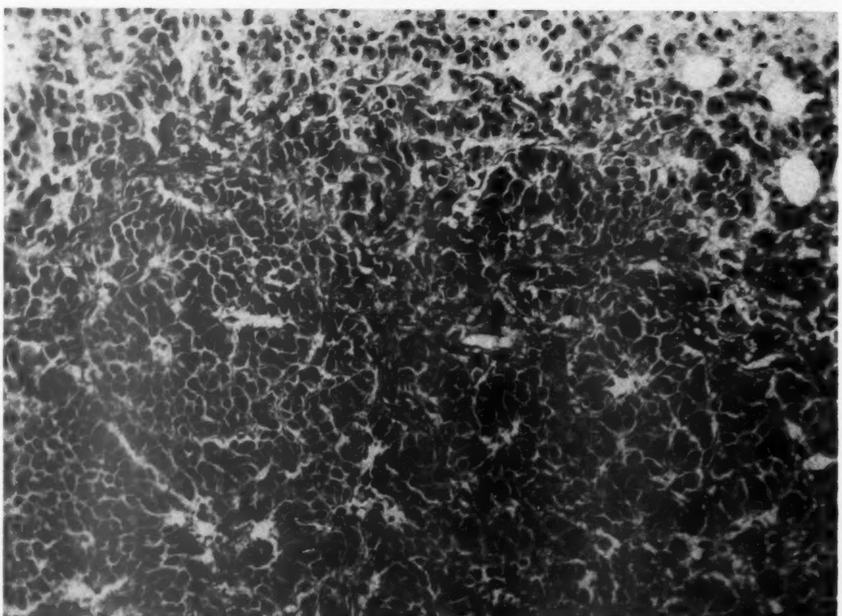

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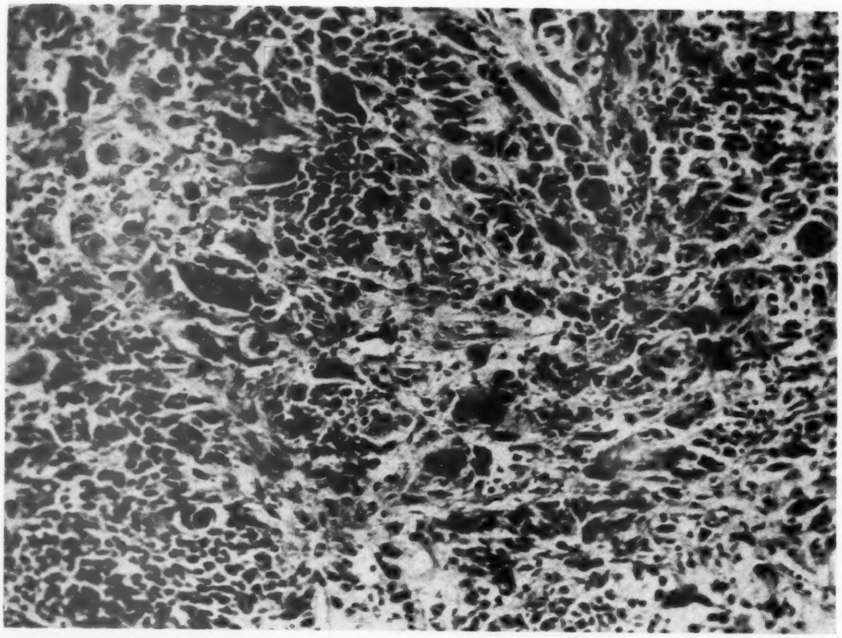
FIG. 20. Ependymoma seen on 13th day after irradiation with single dose of 1200 r. Mixture of bizarre giant tumor cells and ependymal glia. Hematoxylin and eosin stain. $\times$ 185.

FIG. 21. Appearance of ependymoma on fourth day after radiotherapy of 5000 r. Rosettes are absent; individual cells are pale and swollen. Hematoxylin and eosin stain. $\times$ 185.



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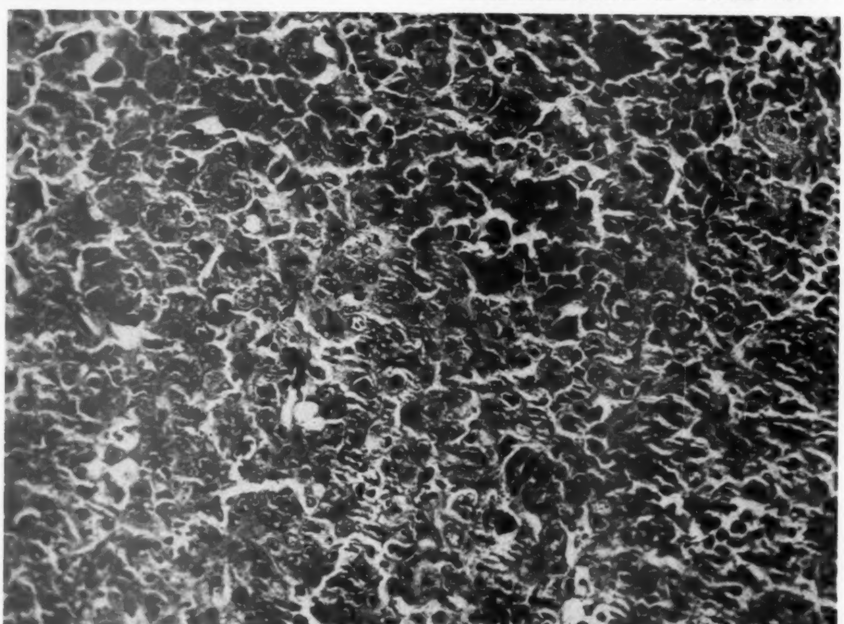

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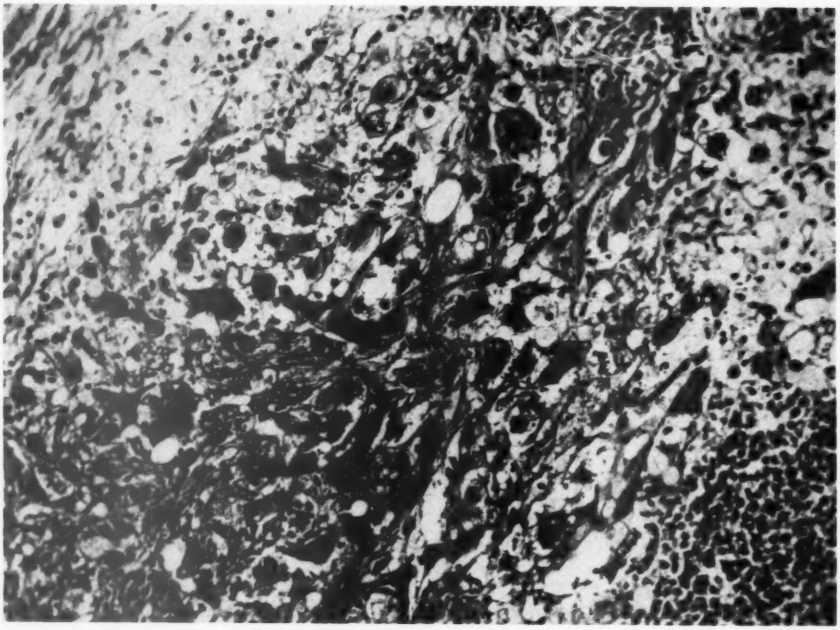
FIG. 22. Ependymoma on 14th day after x-irradiation with 5000 r. There is absence of cells resembling ependyma and in their place are huge tumor gliacytes. Hematoxylin and eosin stain. × 185.

FIG. 23. Twenty days after the x-ray dosage of 5000 r. the ependymoma once again has some identifiable ependymal cells. Hematoxylin and eosin stain. × 185.



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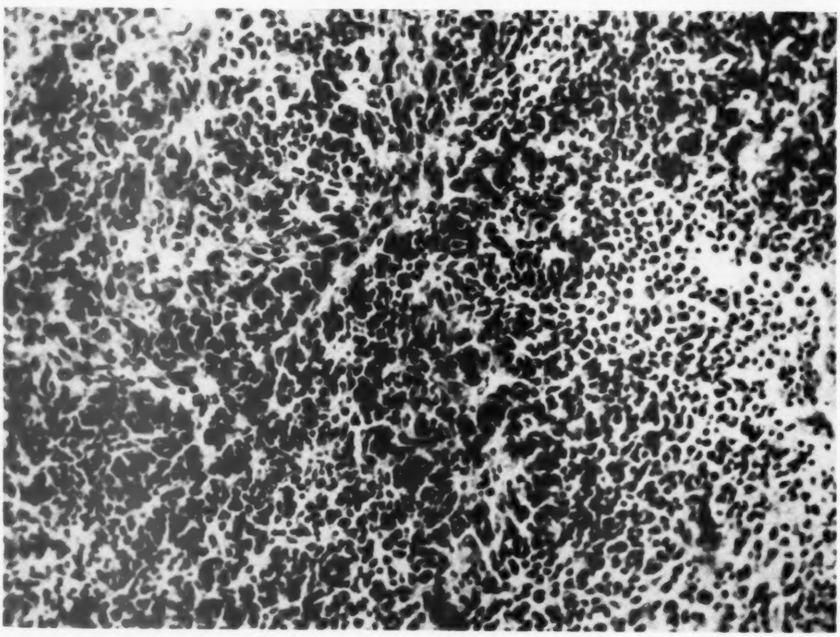

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THE VIRAL RANGE IN VITRO OF A MALIGNANT HUMAN EPITHELIAL CELL (STRAIN HELA, GEY)

III. Studies with Pseudolymphocytic Choriomeningitis Virus General Discussion *

William F. Scherer, M.D.† and Jerome T. Syverton, M.D.
From the Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minn.

The viral range of a strain of human malignant epithelial cells (strain HeLa, Gey) has been shown to include the viruses of poliomyelitis, herpes simplex, pseudorabies, vaccinia, Eastern equine encephalomyelitis, Western equine encephalomyelitis, St. Louis encephalitis, West Nile, and Japanese B encephalitis.[1-6] It is the purpose of this paper to present the results of studies with pseudolymphocytic choriomeningitis virus and to discuss the findings reported in this paper and in the preceding two papers of this series.[5,6]

Materials and Methods

Viruses. Pseudolymphocytic choriomeningitis virus, MacCallum strain, was kindly supplied in 1952 by courtesy of Drs. M. M. Sigel and T. F. McNair Scott. The virus was passed intracerebrally once in mice to prepare a 10 per cent suspension of infected brain tissue. The LD_{50} of this suspension was $10^{-6.6}$ per 0.03 to 0.05 ml.

Lymphocytic choriomeningitis virus, Armstrong strain, kindly supplied by the State of Minnesota Public Health Laboratories was employed as a 5 per cent suspension of infected brain in sterile 5 per cent dextrose in water. The LD_{50} was $10^{-4.6}$ per 0.03 to 0.05 ml. of suspension.

Encephalomyocarditis virus, MM strain, was used as a 10 per cent suspension of infected mouse brain. The LD_{50} was $10^{-9.3}$ per 0.03 to 0.05 ml. of suspension.

Mouse encephalomyelitis virus, FA strain, labeled "Theiler's, FA, 10% br, 2-20-49, NRS-S, 1 cc.," was obtained by courtesy of Dr. C. M. Eklund. The virus employed, contained in 10 per cent suspension from the second mouse passage in our laboratory, had an LD_{50} of $10^{-5.5}$ per 0.03 to 0.05 ml.

The methods for ***viral assay, cellular cultivation, viral propagation,*** and ***photography*** were described in the first article of this series.[5]

Experimental Results

The viruses of lymphocytic choriomeningitis (Armstrong strain), pseudolymphocytic choriomeningitis (MacCallum strain), encephalo-

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Parts I and II, dealing, respectively, with herpes simplex, pseudorabies, and vaccinia viruses, and with encephalitis viruses of the Eastern, Western, West Nile, St. Louis, and Japanese B types, appeared in the preceding issue of this *Journal*. The section on General Discussion of the present paper refers to the three portions of the series.—*Editor.*

† John and Mary R. Markle Scholar in Medical Science.

myocarditis (MM strain), and mouse encephalomyelitis (FA strain) were studied to learn whether each could destroy strain HeLa cells. Only pseudolymphocytic choriomeningitis virus was found to possess a cytopathogenic effect for strain HeLa cells.

Pseudolymphocytic Choriomeningitis Virus

Cytologic Effects of Pseudolymphocytic Choriomeningitis Virus. A cytopathogenic effect of pseudolymphocytic choriomeningitis virus became evident during the first passage of virus in strain HeLa cells. To record these changes, cells infected with virus in Porter flask cultures were photographed.

Experiment 1. The procedure employed for this experiment was similar to that used for the study with Western equine encephalomyelitis virus.[6] Pseudolymphocytic choriomeningitis virus from the eleventh passage in strain HeLa cells (experiment 2) was employed to infect these cultures. The effects of this virus upon strain HeLa cells are presented in Figures 1 and 2.

The destructive effects of pseudolymphocytic choriomeningitis virus for strain HeLa cells are shown in Figures 1 and 2. By the third day after viral inoculation, the polygonal cells had become round and many had conglomerated. Degenerate cells were seen among normal cells (Fig. 1). During the next 2 days of incubation, more extensive cellular damage occurred, resulting in aggregates of dead and degenerate cells (Fig. 2). Cells in parallel but uninoculated control cultures retained their normal appearance.

Multiplication of Pseudolymphocytic Choriomeningitis Virus. Passage of pseudolymphocytic choriomeningitis virus through a series of cultures of strain HeLa cells was tried to learn whether virus multiplication would occur.

Experiment 2. The procedure for this experiment was similar to that for the study of Western equine encephalomyelitis virus.[6] The data from this experiment are given in Table I.

Evidence for the propagation of pseudolymphocytic choriomeningitis virus in cultures of strain HeLa is shown in Table I. Virus, cytopathogenic for strain HeLa cells, persisted through 15 serial passages over an 82-day period. The original virus (LD_{50}, $10^{-6.6}$) inoculated into the first passage had been diluted thereby at least 10^{15} times. The supernatant fluid from the twelfth passage yielded an infectivity end point of $10^{-4.0}$ upon titration in mice and in strain HeLa cultures. Similarly, quantitative measurements by titration in strain HeLa cultures of virus from the fifth, eighth, and ninth passages provided evidence of virus multiplication.

To learn whether the viruses of lymphocytic choriomeningitis

(Armstrong strain), encephalomyocarditis (MM strain), and mouse encephalomyelitis (FA strain) cause destruction of strain HeLa cells, three experiments were carried out with lymphocytic choriomeningitis virus, four with encephalomyocarditis virus, and one with mouse encephalomyelitis virus. Cells were observed after viral inoculation for as long as 21 days. The viruses upon inoculation to cultures yielded as final concentration of brain suspension: 10^{-1} and 10^{-2} for lymphocytic choriomeningitis virus; 10^{-1}, 10^{-4}, and 10^{-6} for encephalomyocarditis virus; 10^{-1} for mouse encephalomyelitis virus. In one trial with a 10^{-1}

TABLE I

Propagation in Vitro of Pseudolymphocytic Choriomeningitis Virus in Cultures of Human Malignant Epithelial Cells (Strain HeLa, Gey)

Number of virus passage	Total days in culture	Cumulative log of dilution of original viral inoculum	Results as indicated by		
			Cytopathogenic effect	Infectivity log of cultural fluid for	
				Mice*	HeLa cells†
Inoculum				6.6	
1	3	1.0	2/2‡		
5	33	5.0	2/2		2.0–3.0
8	57	8.0	2/2		3.0–4.0
9	60	9.0	2/2		4.0–5.0
12	71	12.0	2/2	4.0	4.0
15	82	15.0	2/2		

* The results of mouse titrations expressed as the negative log of the LD_{50}/0.05 ml. of diluted cultural liquid.

† The results of strain HeLa titrations are given as the negative log of the dilution of cultural liquid which, per 0.4 ml., produced a specific viral cytopathogenic effect in a tube culture of cells after 6 to 7 days of incubation at 36° C.

‡ The numerator signifies the number of cultures that showed a viral cytopathogenic effect. The denominator indicates the number of cultures inoculated with virus.

concentration of encephalomyocarditis virus, slight degeneration of cells was observed. However, passage of cultural liquid to other cultures produced no destruction of cells during 8 days of incubation at 36° C. Cellular destruction was not observed in the three other attempts with encephalomyocarditis virus, or in the experiments with lymphocytic choriomeningitis or mouse encephalomyelitis viruses. The significance of these negative findings is attested by concurrent successful propagation of herpes simplex virus, and of pseudolymphocytic choriomeningitis virus. However, since a single strain of each virus was studied, these findings leave open the possibilities that other strains of these viruses can destroy strain HeLa cells. Moreover, since the

medium employed (CHS–10, MS–90), maintains cells at a relatively slow rate of growth and metabolism, strain HeLa cells at a different metabolic level might exhibit a different response to these viruses.

General Discussion

The results of these studies demonstrate that malignant epithelial cells, strain HeLa, Gey, support propagation *in vitro* of the viruses of herpes simplex, pseudorabies, vaccinia, Eastern equine encephalomyelitis, Western equine encephalomyelitis, West Nile, St. Louis encephalitis, Japanese B encephalitis, and pseudolymphocytic choriomeningitis. Seven of these nine viruses regularly destroyed strain HeLa cells. St. Louis encephalitis virus resulted irregularly in cellular destruction even though the cultures were observed for as long as 19 days after virus inoculation, and viral inhibitors from the human serum medium had been removed by a $10^{5.2}$ dilution with balanced salt solution. Japanese B encephalitis virus after four passages failed to affect cellular morphology. The destructive effects of pseudorabies, West Nile, and pseudolymphocytic choriomeningitis viruses were delayed for several days after inoculation. In contrast, the effects of infection by the viruses of herpes simplex, vaccinia, EEE, and WEE were usually seen within 24 to 48 hours with rapid spread to kill all cells. Pseudorabies virus commonly evoked circumscribed foci of degeneration. When small viral inocula were employed, herpes simplex and vaccinia viruses gave rise occasionally to similar foci. Intranuclear acidophilic inclusion bodies of type A were found in cells infected by the virus of herpes simplex or of pseudorabies; cytoplasmic inclusions occurred with vaccinia virus.

Four possible explanations for the absence of cellular destruction by SLE and JBE viruses are worthy of brief discussion. The first three explanations are based on the premise that the process of virus multiplication occurs independently of the mechanism responsible for cellular destruction: (1) The strains of SLE and JBE virus employed for these studies obviously failed to destroy cells; whether this lack of cytopathogenic effect applies to other strains of SLE or JBE virus is not known. (2) The progeny of the original virus, with serial passage, either loses the property of cytopathogenic effect, or selective replacement by non-cytopathogenic clones takes place. The result is an increase in viral population without overt cytologic change. (3) Components of the cultural medium, for example, chicken serum, inhibit the destructive effect of virus, without effect upon viral reproduction. (This explanation is suggested for the lack of cytologic changes in pas-

sages 4 to 6 of West Nile virus, in which serum from a single chicken prevented or delayed destruction of cells even though virus multiplied.) A fourth explanation would assume that viral reproduction results uniformly in cytologic changes; yet so little virus multiplication actually occurs that the resultant destruction of cells goes unrecognized upon microscopic examination. If this explanation were valid, extensive and readily observed cellular destruction would result only when a large quantity of virus is inoculated, e.g., a mouse brain suspension of SLE virus in its first passage, or when the yield of virus from the process of virus multiplication becomes great enough to result in widespread infection and cellular destruction. Evidence in support of each of these explanations is currently being sought experimentally.

Strain HeLa cells readily support multiplication of poliomyelitis virus.[2,3] The propagation of each of the three types of virus was accompanied by progressive destruction of the cells in from 12 to 96 hours. These destructive effects were prevented by employment of the homotypic poliomyelitis antibody, though not by heterotypic antibodies. The many distinctive properties of this stable strain of human epithelial cells for the study of the poliomyelitis group of viruses made it important to learn whether it can be employed advantageously for the detection, propagation, and specific immunologic identification of other viruses infectious for man. The recognition that strain HeLa cells might support growth of a variety of viruses with result in cellular destruction led to the present series of investigations.

The comparative cytopathogenic effects for strain HeLa cells of the variety of viruses studied herein differ from the effect of poliomyelitis virus: (a) The time required for cellular destruction from infection by pseudorabies, West Nile, or pseudolymphocytic choriomeningitis viruses was prolonged over at least from 3 to 5 days in contrast to poliomyelitis virus which requires from 1 to 3 days. (b) Viable cells often remained on the glass surfaces of culture flasks for several days after infection by these viruses had resulted in destruction of a majority of cells. In contrast, infection of strain HeLa cells by poliomyelitis virus progressed rapidly to produce total cellular destruction and detachment of cells from the glass surface. (c) Cells infected by eight of these nine viruses, unlike cells affected by poliomyelitis virus, became round and clumped; yet they retained a distinct, intact outer surface. The "shattered" or "ruptured" appearance of strain HeLa cells that results from infection by poliomyelitis viruses was simulated only by West Nile virus.

For contrast with the destructive effects for strain HeLa cells of

viruses other than poliomyelitis virus, photographs of cells destroyed by poliomyelitis virus are presented in Figures 3 to 6. It can be seen that localized areas of cellular degeneration clear of cells and bordered by round, clumped cells may occur from 2 to 4 days after inoculation of small quantities of poliomyelitis virus (Figs. 3 and 4). These areas appear similar to those observed from infection of HeLa cells by herpes simplex, pseudorabies, or vaccinia viruses. By the fourth day of infection by poliomyelitis virus or within from 12 to 72 hours after inoculation of large quantities of virus, the cells were small, round, irregular in shape, and had a rough, disrupted surface (Fig. 5). Eventually, either no cells or a few degenerate cells remained on the glass surfaces of the culture flasks (Fig. 6).

The relative ease by which strain HeLa cellular cultures can be mass produced has permitted extensive studies with poliomyelitis virus.[2,3] Cultures of strain HeLa cells have been shown to be useful in work with poliomyelitis virus (a) for isolation of virus, (b) for assay of virus, (c) for production of virus, and (d) for the measurement of neutralizing antibodies. Similarly, sufficient evidence has been cited herein to suggest that HeLa cells offer comparable advantages for exploitation in application to many other viruses. For examples, isolation and identification of herpes simplex virus have been successfully carried out by inoculation of strain HeLa cellular cultures with vesicular fluid and a suspension from a human herpetic lesion. Titration of herpes simplex, or of pseudorabies virus, is relatively easy to perform in cultures of strain HeLa, though with current techniques the endpoints may be slightly lower than in mice. Production of virus in quantity can be readily accomplished by the employment of strain HeLa cells. Neutralization tests can be effected with cytopathogenic viruses, viz., herpes simplex, pseudorabies, vaccinia, EEE, WEE, West Nile, and pseudolymphocytic choriomeningitis viruses. Finally, direct observation of cells and of intracellular inclusion bodies is facilitated by the single cells and cellular sheets of single-cell thickness present in cultures of strain HeLa. Thus, strain HeLa cells undoubtedly will be applied to the study of many problems for a variety of virus diseases.

Summary

Pseudolymphocytic choriomeningitis virus was found to propagate in and destroy human cells of a strain derived in 1951 from an epidermoid carcinoma of the cervix (strain HeLa, Gey). Thus, the viral range for strain HeLa cells includes pseudolymphocytic choriomeningitis, herpes simplex, pseudorabies, vaccinia, Eastern equine en-

cephalomyelitis, Western equine encephalomyelitis, West Nile, St. Louis encephalitis, Japanese B encephalitis, and poliomyelitis. Infection by seven of these viruses resulted in cytologic alterations in strain HeLa cells that differed from, yet were similar to, the effects of poliomyelitis virus. The uses of strain HeLa cells for isolation of virus, for titration, for viral production, for assay of neutralization antibodies, and for the study of inclusion bodies were discussed.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

All illustrations are photographs of unstained cells.

FIG. 1. Early evidence of the cytopathogenic effect of pseudolymphocytic choriomeningitis virus is shown by aggregates of round, degenerate cells 3 days after viral inoculation. × 150.

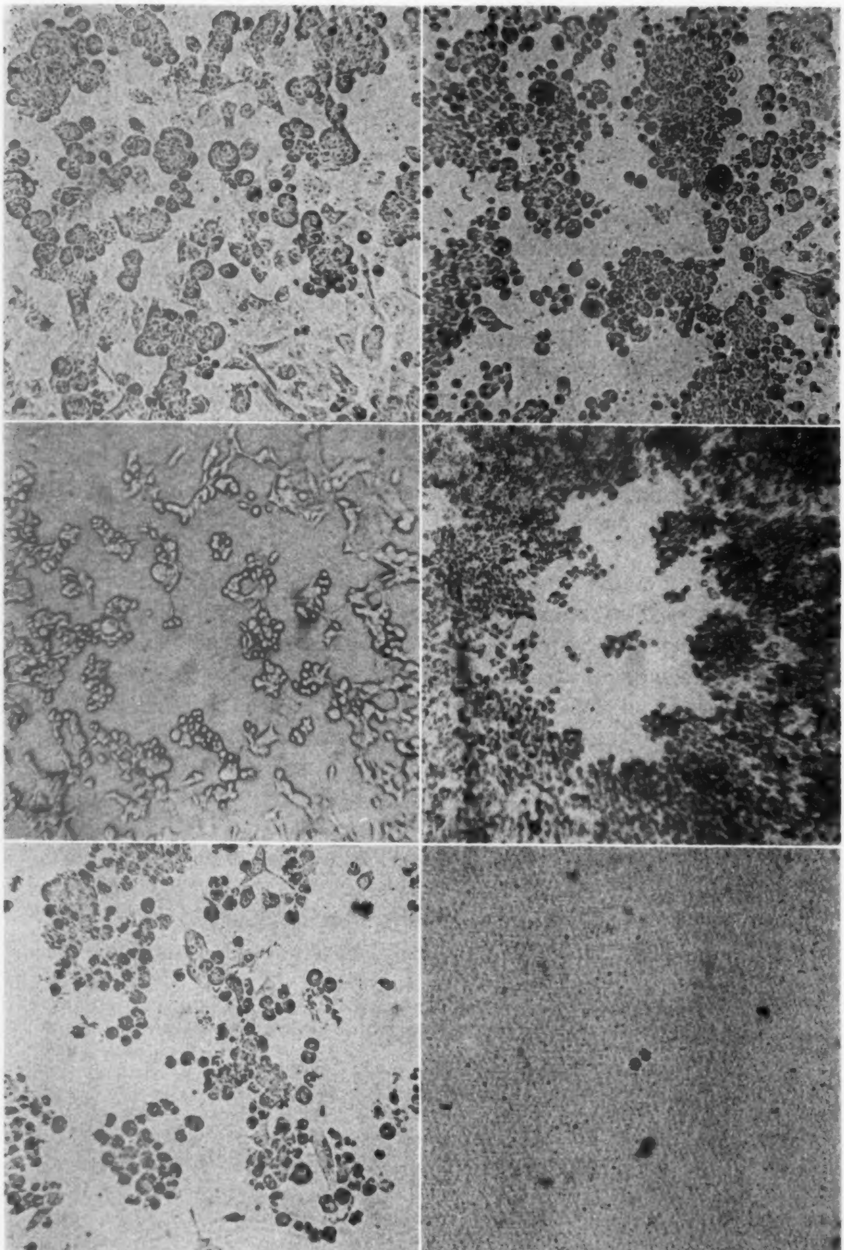
FIG. 2. Nearly total destruction of strain HeLa cells photographed 5 days after inoculation with pseudolymphocytic choriomeningitis virus. × 130.

FIG. 3. For comparative purposes, a small focal area of cellular degeneration in a tube culture of strain HeLa was photographed 3 days after inoculation with a 10^8 dilution of poliomyelitis virus, type 1, Mahoney strain. Of nine cultures, three for testing each dilution, the three for the 10^8 dilution eventually showed complete cellular destruction, but there was none for $10^{8.7}$ or 10^9. × 100.

FIG. 4. A focus of cellular degeneration in a tube culture of strain HeLa photographed 3 days after infection by poliomyelitis virus, type 1, Mahoney strain, in a 10^7 dilution. Total destruction eventually resulted within 7 days from the 10^7 and 10^8 dilutions. × 100.

FIG. 5. "Shattered" strain HeLa cells photographed 1 day after infection by poliomyelitis virus, type 3, Saukett strain. × 150.

FIG. 6. Two round, degenerate cells on the glass surface of the Porter flask illustrate the extensive destruction of strain HeLa cells that had resulted 2 days after infection by poliomyelitis virus, type 3, Saukett strain. × 150.



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5 6

PATHOLOGY OF SWINE EXPOSED TO TOTAL BODY GAMMA RADIATION FROM AN ATOMIC BOMB SOURCE *

John L. Tullis, Cdr., M.C., U.S.N.;† Baldwin G. Lamson, Lt., M.C., U.S.N.R.,‡ and Sidney C. Madden, M.D.‡

From the Naval Medical Research Institute, Bethesda, Md. (Drs. Tullis and Lamson), and the Brookhaven National Laboratory, Upton, N.Y. (Dr. Madden)

The appearance and progression of the lesions occurring in swine after exposure to approximately 700 r. gamma radiation from an atomic bomb source are described in this study. The radiation was spontaneously lethal on the seventh day after exposure to all animals not previously sacrificed.

The earliest lesions were mitotic arrest and necrosis of lymphoid elements, and necrosis of small intestinal epithelium, both seen after 4 hours. Mitotic arrest in hematopoietic cells of the bone marrow appeared at 8 hours and progressed to extensive disappearance of marrow cells by 3 to 4 days after radiation. This may be the most significant histologic basis for death in these animals, because it impaired both antibacterial defenses and vascular reparative mechanisms. Hemorrhage and bacterial invasion were conspicuous in the later deaths.

The possible sequence of tissue damage and repair after atomic bomb radiation in man may be estimated from these observations in swine, because the general level of radiosensitivity, the terminal picture following lethal exposure, and the size of the experimental animal all approximate those of man. The aluminum shielding of the swine, affording its principal protection against thermal and mechanical injury, would tend to limit the extent of this comparison. The terminal picture following exposure to a lethal dose of ionizing radiation has been described for man,[1,2] swine,[3-5] and for many laboratory animals. The sequence of tissue changes has been given detailed study in various species[6-11] and in swine[12] following supervoltage x-radiation. These x-irradiation injuries in swine may well be compared with those of this report following atomic bomb radiation. It will be seen that

* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the view of the Navy Department or the naval service at large.

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† Now at: Cancer Research Institute, New England Deaconess Hospital, Boston, Mass.

‡ Now at: School of Medicine, University of California, Los Angeles, Calif.

the lesions are similar although their severity and the mortality are greater in the group exposed to the atomic bomb.

Materials and Methods

The Landrace cross-breed swine* for this study were farrowed and raised at the test site, thus assuring fully acclimatized animals. Their ancestral origin was the same as that of the swine used in control experiments with supervoltage x-radiation at the Naval Medical Research Institute.[12]

The swine were weaned at 9 weeks and placed on a diet of tankage, fish meal, soybean meal, linseed meal, alfalfa meal, and mineral mixture in a proportion recommended by the U.S. Department of Agriculture Center, Beltsville, Md. It was offered *ad libitum*. They were raised under sanitary conditions and received hog cholera serum-virus treatment 3 weeks before weaning and were given booster doses 25 days before the experiment began.

The 32 test swine were healthy animals, 3½ to 5½ months old, ranging in weight from 63 to 78 lb., with a mean weight of 70.3 lb. 2 days before they were radiated (Table I).

On the morning before radiation 16 female and 16 male swine were placed in the aluminum liners of their exposure cages and then inserted in fixed, fireproof, blastproof, aluminum cylinders at the test station. Adequate water was provided but no food.

All animals were approximately equidistant from the bomb and the exposure dose approximated 700 r. total body gamma radiation. An attempt to control the side of the animal facing the bomb was thwarted by the ability of some to reverse their positions in the cylinders.

As soon as possible after the bomb was detonated, the test animals were recovered. All of the swine were apparently in good condition and healthy on recovery and still had water available. Return to their pens was complete by 12 hours after radiation, thus terminating their confinement in the aluminum cylinders at a maximum of 36 hours.

One female and one male swine were killed by electrocution 4 hours after radiation, using a 220 volt current administered by battery-clamp contacts on skin moistened with electrolyte. Eleven more pairs of test swine were similarly killed at intervals up to and including 146 hours after radiation. The remaining 8 animals died spontaneously of their radiation injury between 143 and 169 hours after the exposure. The

* Ancestral stock obtained from the Animal Husbandry Experiment Station, Bureau of Animal Industry, Agricultural Research Center, United States Department of Agriculture, Beltsville, Md.

exact schedule of sacrifice by electrocution and the times of spontaneous deaths in the 32 test swine are reproduced in Table I, but elsewhere throughout this report for simplicity the times of death are

TABLE I

Sacrifice Schedule, Spontaneous Time of Death, and Weight Loss Following 700 r. Radiation from Atomic Bomb

Swine no. and sex	Time of death after radiation	Weight: 2 days before radiation	Weight: At death	Weight: Decrease
	hr.	*lb.*	*lb.*	%
418F	4	66	55	17
477M	4	71	58	18
430F	7½	70	54	23
475M	7½	73	59	19
340F	15½	67	59	12
362M	15½	65	56	14
338F	25½	69	65	6
428M	25½	71	61	14
452F	36½	72	68	6
471M	36½	67	61	9
327F	49½	65	59	9
472M	49½	71	58	18
367F	60½	70	64	9
382M	60½	64	58	9
429F	73½	75	63	16
462M	73½	63	55	13
328F	84½	70	65	7
411M	84½	69	62	10
409F	97½	70	52	26
352M	97½	77	66	14
330F	123	76	60	21
360M	123	75	65	13
324F	146½	74	65	12
468M	146½	71	61	14
*476F	143	68	55	19
*384M	146	73	56	23
*393M	152	69	55	20
*405F	157½	66	52	21
*425M	159	73	58	21
*373F	161½	67	55	18
*323F	166	78	62	21
*364M	169	77	59	23

* Spontaneous death.

referred to by the following approximations: 4, 8, and 16 hours; 1, 1½, 2, 2½, 3, 3½, 4, 5, 6, and 7 days after radiation.

Representative tissue blocks were fixed in 10 per cent neutral formalin. After fixation and trimming, the formalin-fixed tissue blocks were imbedded in paraffin, sectioned at 4 to 6 μ, and stained with hematoxylin and eosin and Giemsa's stains as indicated.

At necropsy, aerobic and anaerobic cultures of the heart's blood were made.

Ten swine of the same animal colony, 5 males and 5 females, were subjected to loading, handling, transportation, and the 36-hour confinement in exposure cylinders; in short, they sustained all manipulations experienced by test animals except the radiation. They lost in body weight during the 36-hour confinement period and fast from 9.2 to 25.6 per cent, a mean of 14.9 per cent, but regained the lost weight within 24 hours after having access to unlimited food. These control-study swine were electrocuted, necropsied, and their tissues processed and studied in the same manner as the test swine.

Experimental Observations

Clinical Course

On return to the animal runs after exposure to approximately 700 r. total body gamma irradiation from the blast, the swine were active, hungry following their 36-hour fast, and irritable, but otherwise appeared to be in good condition. After 24 to 48 hours they became less irritable and, in fact, began to develop the lethargy that was a prominent feature during the later and more severe stages of the response to radiation. At the same time, increased thirst, loss of appetite, and loose stools were noted. By the fourth day most of the swine had liquid stools, ate very little, but drank water copiously, and were content to spend most of their time lying down in the shade. In some the stools became watery on the fifth day but in others the diarrhea abated somewhat. Soft formed stools were noted in all surviving swine from time to time thereafter until the 24 hours preceding spontaneous death.

In those which died spontaneously during the seventh day, stupor, elevated body temperature, white frothy spittle, and rapid respirations were seen terminally. The hind quarters of these animals were usually wet with liquid feces and their flanks and eyeballs were sunken and retracted. Rigor mortis occurred within 2 to 3 hours after death.

The alteration in physical appearance and clinical signs in the 24 hours preceding spontaneous death was striking. The change from the obviously but not alarmingly sick ambulatory swine to the moribund animal was rapid and without externally apparent explanation.

Gross Lesions

Early Changes

Radiation-induced tissue changes developed gradually during the first 4 days following exposure, making their exact time of onset difficult of gross recognition. Small hemolymph nodes and scattered elec-

trocution-induced pleural petechiae in control animals added to the difficulty in determining the onset of hemorrhages related to radiation injury. During the first few days alterations in the appearance of the thymus, bone marrow, and bowel were noted. These will be described in the sequence in which they were first observed.

Reduction in size of the thymus was first noted 1½ days after radiation and was conspicuous after the third day. The thymus became softer and brownish as progressive decrease in size occurred. Regeneration was not detected grossly during this study. The bone marrow was drier and lighter red after the second day and assumed these characteristics with increasing degree as time advanced. Changes in the bowel occurred sporadically. Mild congestion of the small and large bowel was generally present during the first 1½ days after exposure. To what extent radiation contributed to these changes is debatable in view of the mildly congested bowel frequently observed in electrocuted non-radiated swine. One animal killed at 1½ days showed a 10 by 3 mm. area of increased congestion in the ileum, possibly a result of radiation. At 2 days another had prominent dark red discoloration of the upper ileum of greater intensity than seen in control animals. At 2½ days one showed a fiery red discoloration of the entire duodenum, jejunum, and cecum. These areas were covered with adherent mucus and proved on microscopic examination to be acutely inflamed. Additional prominent areas of congestion were present in the ileum and colon at 3½ days, and all 4 animals sacrificed at the end of the fifth and sixth days showed more extensive congestion of the gastro-intestinal tract, with most marked involvement of the duodenum and upper jejunum. Hemorrhagic streaks appeared in the mucosa of the jejunum and ileum in one animal, and multiple mucosal petechiae were common throughout the small and large bowel in another.

Extension of Gross Lesions Prior to Death

Spontaneous deaths were imminent in the swine sacrificed at the end of days 5 and 6, and gross pathologic alterations were more widespread and more uniform. Pale yellow, clear, peritoneal fluid had accumulated in small amounts, and in one animal a thin layer of fresh fibrin covered the serosal surface of loops of bowel. Epicardial and endocardial petechiae were present. The myocardium was more flabby and cloudy in cross section. Edema of the subserosa of the gallbladder distended the wall up to 1.0 cm. in thickness. The spleen appeared more dry, flabby, and thin. Malpighian corpuscles were no longer visible. Kidneys showed hyperemia and pale swollen cortices. Gonads of both sexes were hyperemic and cyanotic.

The thymus decreased in size to the extent that it became difficult to locate. Peripheral and visceral lymph nodes did not show a companion decrease in size, however, probably because of increasing hemorrhage into these structures. At the end of day 5 and thereafter, the extent of hemorrhage into lymph nodes exceeded by two- to threefold the amount seen in lymph nodes of control animals. (See discussion under Incidental Findings.)

Spontaneous Deaths

The gross pathologic changes observed in swine dying spontaneously were qualitatively similar to those described for animals which were sacrificed at the end of days 5 and 6. There was, however, conspicuous extension and progression of these lesions during day 7, in which all 8 spontaneous deaths occurred. More specifically, these 8 swine showed evidence of severe dehydration and recent weight loss, sunken eyeballs, and concave flanks. Lividity of abdominal skin was pronounced in one. Small, shallow ulcerations appeared in the buccal mucosa in two swine (Fig. 1). Similar ulcers were found along the dorsolateral margins of the tongue in another. Hemorrhage about the lateral angle of the mouth extended into muscle and subcutaneous tissue in one swine. In all 8 animals, the tonsils were sharply demarcated from adjacent tissue by their dark brown to black discoloration, produced by hemorrhage and hyperemia which were clearly visible through the intact pharyngeal mucosa (Fig. 2).

Unusually high body temperature was noted in animals necropsied shortly after death. Blood was observed to clot rapidly. The pleural cavities did not contain excess fluid and pleural surfaces were smooth, gray, and glistening except where discolored by subserosal hemorrhages ranging from petechiae to large ecchymoses 8 to 10 cm. in diameter (Fig. 3). These hemorrhages often extended into the loose periaortic connective tissue of the posterior mediastinum and sometimes into the diaphragm. The pericardial cavity contained a slight excess of clear yellow fluid in 2 animals. Similar fibrin-containing fluid, measuring not more than 100 cc., was found in the peritoneal cavities of 4 swine. A thin layer of fibrin was loosely adherent over random loops of bowel in 2 other animals which, however, did not show an accumulation of peritoneal fluid. Peritoneal surfaces elsewhere were smooth and glistening, but hemorrhages were seen occasionally in the subperitoneal spaces.

The hearts were dilated and the myocardium was softer, more flabby, and had a pale turbid appearance. Petechiae and other small

hemorrhages were observed beneath both epicardial and endocardial surfaces.

A striking change was present in the lungs, which were covered with extensive subpleural hemorrhages of all sizes and were made very heavy by hemorrhagic edema fluid (Fig. 4). In one animal a large pulmonary hematoma was formed by extensive bleeding into an emphysematous pleural bleb. A small recent infarct was seen in one instance.

The livers after spontaneous death were more friable and congested and assumed a more yellowish red-brown color than in the sacrificed swine. Edema of the gallbladder wall increased and petechiae appeared beneath the mucosal surface. Bile was in scant amount in most animals and was pale honey-yellow.

Progression of changes in the spleen was not grossly evident. The pancreas was softer than previously noted. Several animals possessed swollen adrenal glands which were predominantly yellowish brown when sectioned.

Petechiae were noted on the cortical surfaces of the kidneys, in the parenchyma on cut section, and in the mucosal lining of the pelves and calyces (Fig. 5). The organs as a whole appeared swollen and congested in the medullary portion with a paler grayish yellow cast in the cortex. The bladder mucosa contained small hemorrhages in one animal. Others showed mucosal hyperemia. Genital organs showed no gross evidence of disease beyond the hyperemia and cyanosis already described.

The thymus remained very small and was soft and light yellowish brown. Hemorrhage was noted in one thymus. Lymph nodes in these swine were generally congested or hemorrhagic to a pronounced degree. An occasional gray node could be found, but the great majority in all locations were reddish black, soft, and on section distended with blood.

Recent bleeding into joint spaces was observed in 2 of the 8 animals which died spontaneously (Fig. 6). Bone marrow remained dry, light red, and scant.

The alimentary tract with the exception of the esophagus was the site of extensive damage in all 8 animals dying spontaneously, in contrast to the relatively minor degree of hyperemia and hemorrhage in the sacrificed swine. The mucosa of the stomach was edematous, generally hyperemic, fiery red, and speckled with discrete hemorrhages ranging in size from petechiae to large ecchymoses. These changes usually were more prominent in the pyloric region. Adherent green

and yellow mucus over areas of damaged mucosa in the pyloric region offered vivid contrast to the deep fiery red of the intact surface (Fig. 7).

The duodenum also was intensely hyperemic and generally deep red. It likewise showed extensive bleeding into the mucosa forming a variety of reddish black mottled and speckled patterns. The hyperemia and hemorrhage of the small bowel were usually most intense in the proximal portion of the duodenum, diminishing in intensity in the jejunum and ileum (Fig. 8). In one animal, at the site of an intussusception, jejunal bleeding into the mucosa produced a large hematoma of cylindric form, 2 to 3 cm. in diameter and 8 to 10 cm. in length, which completely occluded the lumen (Fig. 9). Occasional ulcers were seen in the small bowel with their bases covered by an adherent, tenacious, greenish gray membrane. These were oriented usually along the axis of the bowel and were most frequent along the mesenteric border. Ulcers were not observed in portions of bowel uninvolved by hemorrhage.

Similar congestion, hemorrhage, and ulceration were found in all parts of the large bowel, the cecum being particularly susceptible (Fig. 10).

Incidental Findings

Frequently, scattered pleural and parenchymal lung petechiae, and occasionally, a small amount of edema fluid and partial atelectasis occurred in the brief period of anoxia during electrocution, as heart action commonly persisted beyond cessation of respiration. In addition, all visceral organs were mildly congested.

One lymph node from each of 3 test animals on section showed cystic, clear fluid-filled spaces, or yellow granulomatous nodules—changes of uncertain origin believed to be unrelated to radiation injury. The majority of the lymph nodes in control animals were small, gray, moist, firm, and had distinctive nodal markings in the cortex on cut section. Many nodes, however, contained recent hemorrhage, producing a pink to dark reddish black discoloration and, on section, alterations in appearance ranging from accentuation of the blood-filled cortical sinuses to complete obliteration of normal lymph node architecture. These hemorrhagic nodes were not confined to any one locus but were found in the mediastinal, mesenteric, and inguinal regions. Their presence in control swine is not explained. Small hemic nodes in the periaortic areas were numerous also.

Many swine from both test and control groups showed conspicuous localized disease involving the ileocecal valve and terminal ileum. Here the mucosa was redundant, thickened, indurated, and irregularly

elevated by cystic dilatations within the mucosa, which were filled with yellow-green granular, moderately inspissated, necrotic material. Such necrotic material frequently was exposed and protruded into the lumen through small ulcers in the mucosal surface. These changes were considered to be residual effects of previous specific enteritis. They are a common finding in swine.

TABLE II

Organ Weights in Relation to Time after Irradiation and in Control Swine as Per Cent of Total Body Weight × 100

Animal group	Heart	Lungs	Liver	Kidneys	Brain	Adrenal glands	Spleen	Testes
Irradiated								
(a) Sacrificed; time after irradiation, hours								
4	44,57	73,81	290,310	59,77	28,30	0.91,1.10	15,15	9.1
7½	31,37	56,71	310,330	60,71	26,29	1.30,1.30	15,17	11.0
15½	34,40	63,75	280,310	61,65	28,33	1.00,1.40	12,12	14.0
25½	36,37	65,71	280,310	59,61	27,27	1.10,1.10	11,12	14.0
36½	37,41	65,67	270,270	64,65	22,29	0.55,0.86	11,14	8.7
49½	41,46	76,80	320,330	68,78	25,31	0.80,1.30	13,20	11.0
60½	33,38	76,95	280,300	35,57	26,27	0.61,0.86	8,12	19.0
73½	35,38	60,61	300,380	65,70	27	1.10,1.10	10,13	11.0
84½	34,44	36,60	260,310	61,72	27,29	1.00,1.30	10,11	11.0
97½	32,40	68,75	240,280	62,80	26,29	1.10,1.40	7,10	17.0
123½	37,41	76,82	260,390	59,100	31,33	1.10,2.50	8,10	16.0
146½	36,44	63,68	270	57,72	27,27	1.10,1.40	8, 9	7.9
(b) 6 spontaneous deaths, 143–169 hours after irradiation								
Weight:								
Range	37–44	100–270	290–420	56–74	28–32	1.30–2.50	11–17	10–25
Mean	40	173	350	62	30	1.8	14	16
Control								
5 young	20–50	31–95	130–370	29–57	31–36	1.00–1.40	10–13	7.9– 9.8
5 older	17–41	50–64	210–280	40–62	11–22	0.57–0.85	10–14	23 –36

Another test animal had a hemorrhagic, ulcerative cystitis that was believed not to be related to radiation injury.

Organ Weights

The ages of the 10 control swine and 30 of the 32 irradiated swine, for which organ weights are available, are given in Table II. The control swine are divided equally between two age groups, whereas the test swine are grouped according to their mode of death. Only 3 of the serially-sacrificed group were older than 140 days. The range as to age of the groups presented in Table II is shown in Table III.

Weight changes of large magnitude were seen only in the lungs of swine which died spontaneously. Almost a threefold increase in mean lung weight, from 0.61 per cent of total body weight in the controls to 1.73 per cent of total body weight in the animals which died naturally, was noted. The changes in splenic weight were smaller, with

TABLE III
Range in Ages of Irradiated and Control Swine

Animal group	Age range in days	Animal group	Age range in days
Irradiated		Control	
Sacrificed	107–166	5 younger swine	83–86
Spontaneous death	126–138	5 older swine	158–172

either a normal or slightly increased size for 2 days after irradiation followed by a gradual decrease in size during the fifth and sixth days. Spleens from animals which died spontaneously showed a terminal increase in size, their weight averaging 0.14 per cent of total body weight while the average splenic weights of serial-sacrificed animals during the last 2 days averaged only 0.09 per cent of total body weight.

The kidneys from both the sacrificed and the spontaneously dying groups were generally larger than the kidneys from control swine. The mean weight of kidneys from all irradiated swine was 0.65 per cent of total body weight as compared with the mean value of 0.51 per cent of total body weight found for the kidneys of the control animals. The livers from irradiated animals averaged 3.09 per cent of total body weight while in the control swine they averaged 2.65 per cent of total body weight.

The age of the irradiated animals must be considered particularly when weights of brain, adrenal glands, and testes are compared with control values. As shown in Table II, the brain and adrenal glands from older control animals were normally a smaller proportion of total body weight than in the younger controls. On the other hand, a relative increase in testicular size in the older control swine was evident. The adrenal glands of irradiated animals were apparently somewhat larger than would be expected normally for their age and this increase was more prominent late in the serially sacrificed group and in those dying spontaneously. Lack of weights of brains, adrenal glands, and testes from normal control swine of the same age as the majority of the radiated swine makes interpretation of these data hazardous.

Hearts from radiated swine did not appear to be significantly altered in size. Weight changes were not influenced by sex.

Microscopic Observations

General Remarks

The pattern and the extent of the microscopic lesions in the organs of gamma ray irradiated swine were quite similar to those of the lesions seen in swine exposed to 600 r. total body x-radiation.[12] Lymphocytes, erythroblasts, myeloblasts, spermatogonia, and selected cells of the intestinal epithelium showed the earliest and the most extensive morphologic changes. There was an arrest of mitotic activity, except in the germinal cells, in the animals observed at 4 hours, with a partial resumption of activity in the swine examined 8 hours after irradiation. After day 1, mitotic figures again became progressively infrequent but did not disappear entirely. The primordial cells, such as reticular cells of the reticulo-endothelial system and indifferent cells of the testes, and highly differentiated cells, with the exception of the bowel epithelium, showed little or no morphologic change during the pre-hemorrhagic stage of the disease. Spontaneous death followed so soon after the onset of the hemorrhagic phase that only rarely was there sufficient time for the development of nutritional or pressure necrosis in the cells involved in a hemorrhagic area. The extent of the necrotic changes in animals dying spontaneously was, therefore, about the same as that in the last animals killed at the end of day 6.

In the serially sacrificed swine there were no significant histologic changes in skin, heart, lungs, liver, pancreas, kidneys, ovaries, pituitary body, adrenal glands, urinary bladder, gallbladder, tongue, thyroid and salivary glands, bone, smooth muscle, striated muscle, peripheral nerves, or fat. The teeth, eyes, brain, and spinal cord will be the subjects of further study and separate reports. The lymphoid, hematopoietic, gastro-intestinal, and testicular tissue changes are discussed here.

In the 8 swine which died spontaneously, in addition to changes in the radiosensitive cells as described for the killed animals, edema, capillary dilatation and engorgement, hemorrhages, and bacterial colonies were found widely scattered throughout the body.

Lymphoid Organs

The spleen, lymph nodes, solitary lymph nodules in the bowel, and the tonsils reacted in a similar fashion in the male and female swine killed simultaneously at any given hour after irradiation. The thymus

deviated slightly from the pattern of the other lymphoid tissues as will be pointed out. The histologic features of the spleen, which serves as the prototype for the lymphoid organs at intervals after irradiation, are illustrated in Figures 11 to 18.

In all lymphoid tissues observed in the first 2 swine killed 4 hours after irradiation, destruction of the small lymphocytes was already well established. Fragmented nuclear débris was particularly abundant in the centers of the malpighian corpuscles, in the centers of the lymph follicles in the lymph nodes, and in the tonsils. In the thymus the débris was fairly evenly scattered and distributed throughout the dense lymphoid tissue.

The destructive process had spread to the pulp and reached the maximum level of organ-wide damage observed in the spleen, nodes, and tonsils of the animals killed 8 hours after irradiation. In later observations, the débris gradually was reduced to an amount considerably less than that seen earlier. The destructive process in the thymus continued to increase until a maximum was noted in those swine killed at 16 hours. Later observations showed less destruction until, by the end of 1½ days after irradiation, further destruction of the thymic lymphocytes appeared only rarely. Phagocytosis of débris was in progress in all lymphoid organs of the animals killed at 4 hours. Necrotic material accumulated in the thymus faster than phagocytes could remove it for about 1½ days and for 1 day in the other lymphoid organs, when the process of removal was nearly completed.

After 1 or 1½ days, the lymph follicles were depleted of lymphocytes, leaving the large reticular cells in the center with only a sparse sprinkling of lymphocytes around the periphery. The pulp of the lymphoid organs also showed a marked reduction in the number of lymphocytes present except in the thymus, where the shrinkage of the organ produced the effect of an almost normally dense lymphocyte population in the contracted tissue that remained, in spite of the great reduction in absolute number of lymphocytes.

In addition to the destruction and decrease in total numbers of lymphocytes, a mild infiltration of polymorphonuclear neutrophils appeared during the period of cell destruction. The partial replacement of the destroyed cells by plasma cells was another striking feature. The latter cells became fairly numerous after about 16 hours with what was probably an absolute, as well as a relative, increase. Eosinophils, which are normally abundant in the lymph nodes of swine, were seen throughout the period of observation, although they tended to diminish in those sacrificed late in the series and in those dying spon-

taneously. A similar decrease in eosinophils was found in spleen and tonsils, although a much greater reduction was noted in the spleen than in the other two organs. Multinucleated cells, usually with two or three nuclei and resembling Reed-Sternberg cells, were found occasionally in all lymphoid tissues, beginning about 16 hours after irradiation. These continued to appear during day 7 in the spontaneous deaths but were never numerous.

A period of quiescence with relatively little change in the lymphoid tissue was seen in the thymuses of swine killed 1½ days after radiation and in the other lymphoid organs after 1 day. However, after day 2 lymphocytes began to reappear in the follicles of the spleen and of the lymph nodes. This was a slowly progressive process toward the restoration of normal structure although that goal was never achieved. The replacement of lymphocytes in the spleen was more complete than in the lymph nodes. In the tonsils, repopulation of the lymphocytes did not begin until after day 3 but it then proceeded relatively rapidly. This process was difficult to trace in the thymus because of the tendency for cell density to remain constant as the total organ shrank or expanded.

In animals with spontaneous death on day 7, marked changes took place in a short period of time. Superimposed on the picture of lymphoid regeneration were dilatation and congestion of the small vessels, growth of bacterial colonies, and moderate edema in the splenic pulp. Apparently occurring simultaneously or following in a few hours were multiple small hemorrhages. Death usually occurred within a few hours and before the red blood cells had disintegrated or had been digested by phagocytes. Congestion, hemorrhage, and bacterial implantations were seen in all swine which died spontaneously, yet in 2 killed on day 7, after some spontaneous deaths had occurred, the lymphoid organs showed none of these changes.

Bone Marrow

Red marrow was studied from the sternum, lumbar vertebrae, and both femurs of all animals and was found to respond in a similar fashion in all four locations. Marrow from the left femur was indistinguishable from marrow from the right femur, which was an indication that the dose administered to the animals was fairly uniform in spite of tissue depth and density.

In general, the reaction in the marrow was one of progressive reduction in cellularity. No injury was apparent in the swine killed 4 hours after radiation (Fig. 19), but at 8 hours (Fig. 20) a definite

decrease in the total number of hematopoietic cells was noted. This decrease occurred first among the erythroblasts, but by 16 hours (Fig. 21) the myeloblasts were also beginning to disappear. Blast cells could not be demonstrated with certainty after day 2 (Fig. 22).

The intermediate red blood cell elements dropped off rapidly after 16 hours, but a few myelocytes persisted in the last animal at the end of day 7. A few intermediate to mature polymorphonuclear leukocytes were seen in the marrow sections up to 2½ days after radiation.

Megakaryocytes were abundant in the sections from the pair killed after day 2 (Fig. 22) although only one happens to be included in the field of Figure 22. A day later many had disappeared (Fig. 23), and after 4 days (Fig. 24) only a few remained in each section and these had an abnormal appearance.

The continued progressive depletion of hematopoietic elements is illustrated in the photomicrographs (Figs. 25 and 26). The areas photographed were chosen to represent the most cellular fields available in sections of the sternum. Regeneration of marrow elements was not observed during this study.

Alimentary Tract

In the oral cavity the buccal and gingival mucosae and the tongue bore shallow ulcers late in the course of the disease. These were characteristically sharply demarcated and probably had originated at points of trauma incident to chewing. Bacterial colonies were found in the ulcerated areas. The lack of inflammatory response to the necrotizing process and the normal appearance of adjacent tissue were two outstanding features.

The esophagus appeared normal throughout the 169-hour observation period.

Excepting the depletion of lymphoid tissue in the lamina propria and the submucosa, the stomach sections (the mid-portions of the lesser and greater curvatures were studied routinely) showed very little change until the preterminal stage was reached, when hyperemia of the submucosa was apparent. In those swine which died spontaneously there were small hemorrhages into the submucosa and mucosa. Occasionally the superficial epithelium was lifted away and there was hemorrhage into the lumen of the stomach. At the beginning of day 4 animal 462 had a mild gastritis characterized by a sparse, generalized infiltration of the mucosa of the greater curvature with polymorphonuclear neutrophils and eosinophils. This was not associated with necrosis of the epithelium. A similar reaction was seen in the mucosa of the lesser curvature in swine 324 at the end of day 6.

Routine sections were made from the proximal and distal portions of the duodenum and from four levels at 10-ft. intervals in the jejunum-ileum. The mucosal and submucosal glands in the duodenum showed minimal or no change. Rarely the epithelium in a deep mucosal gland was necrotic, but normal glands were the rule. However, in the small intestine below the duodenum, necrosis of the epithelium of the deep glands was seen 4 hours after radiation (Fig. 27). The injury was associated with a polymorphonuclear leukocytic infiltration in the wall and lumen of the involved gland and in the interglandular stroma. This leukocytic invasion had lessened at 8 hours and was virtually gone in the swine killed 16 hours after irradiation. Deep glandular necrosis was most pronounced in the upper ileum (30 ft. distal to the duodenum), but was found also to a lesser degree in the small intestine above and below this level. Necrosis of these glands in the small intestine was found more frequently in the swine killed after 8 hours (Fig. 28), but by 16 hours this was rarely seen and after day 1 recovery was complete (Fig. 29).

In general, the lymphocytes of the lamina propria were destroyed early and rapidly in line with lymphocytic tissue elsewhere in the body. This had the effect of making the stromal and plasma cells of the lamina propria stand out prominently. After day 1 large numbers of plasma cells had appeared in the lamina propria, particularly in the duodenum (Fig. 29).

Sections of small bowel from one animal killed at 2½ days and thought to present gross changes related to radiation injury, showed hyperemic vessels and infiltration of the lamina propria with neutrophilic polymorphonuclear cells. Near the time of spontaneous death the small-bowel mucosa and submucosa frequently contained hyperemic blood vessels. This feature became more pronounced and widespread in the animals which died spontaneously. The congested areas sometimes showed focal hemorrhages extending into the surrounding tissue and into the lumen of the bowel. Erosion of surface epithelium or shallow ulcers were usually associated with hemorrhage into the lumen.

The ulcers of the ileocecal valve and terminal ileum, which have been described under Gross Pathology, Incidental Findings, appeared microscopically to be of long standing. The bases of the ulcers were composed of fibrous tissue with an extensive acute and chronic inflammatory cellular infiltration. Amorphous necrotic material filled the craters of the ulcers.

Necrosis of the glandular epithelium of the large intestine was similar in type to that seen in the small intestine, but the reaction was

slightly delayed and it occurred relatively infrequently. The lymphoid tissue became depleted as it did elsewhere in the body. During days 3 and 4 acute colitis appeared sporadically. This was characterized by congested blood vessels, edema of the wall, and a polymorphonuclear and eosinophilic leukocytic infiltration of the mucosa. After day 4 mucosal hyperemia was very common. In the swine killed at the end of day 6 and in those dying spontaneously on day 7, hemorrhages into the mucosa and sometimes into the lumen of the bowel were seen (Fig. 30).

Testes

The testes of most of the swine were immature. They contained large numbers of peripherally located indifferent cells (primordial stem cells) and only a few spermatogonia and spermatocytes. Lumina had not yet formed in the tubules. In the few swine which had reached fuller sexual maturity, spermia were found in some of the tubules. The indifferent cells appeared to be normal in all of the swine and the spermatocytes and spermia, where present, showed little or no evidence of radiation injury. However, as early as 4 hours after irradiation there was an increase in fragmented necrotic cells in some of the tubules. The incidence of dead cells was further increased in the swine killed at 8 hours, but decreased thereafter to reach a normal level by day 3.

The most striking change occurred in the spermatogonia beginning at 1½ days, when a decrease in their number was first noted. After this, there was rapid depletion of spermatogonia although they were never completely exterminated. Sertoli cells and Leydig cells were morphologically unharmed. Late in the course of the disease, hyperemia of blood vessels and hemorrhage into the interstitial tissue were noted.

It is interesting that the evidences of radiation injury were fairly extensive in the testicle but were minimal or absent in the ovary. Both male and female gonads may be difficult to evaluate for radiation injury, but the difference in relative sensitivity was unquestionable at this dose level.

Lungs

In the 24 swine killed by electrocution, atelectasis, occasional petechiae, small patches of fluid-filled alveoli sometimes containing large mononuclear cells, and constricted bronchioles were frequently found. Interpretation of radiation injury in these lungs was impossible. In the swine which died spontaneously, hyperemia of the alveolar vessels, many of which contained bacterial masses, and extensive areas of pulmonary edema and hemorrhage involving both alveoli and

bronchi were observed (Fig. 31). The extent of the lung involvement was particularly impressive in the late deaths.

Other Organs

Hemorrhagic and bacterial lesions comparable to those in the lung appeared throughout the body in the animals which died spontaneously (Fig. 32).

Bacteriologic Findings

Aerobic and anaerobic cultures of the heart's blood from 19 of 23 sacrificed swine showed no growth after 5 days of incubation. One culture on day 3 was contaminated; two aerobic cultures were positive on day 5, and one was positive on day 6. The aerobic cultures contained coliform organisms. Aerobic and anaerobic organisms were cultured from both of 2 swine which died spontaneously.

Discussion

Comparison with X-Irradiated Swine

The type and the onset of lesions noted in the 32 swine exposed to approximately 700 r. total body gamma radiation were similar to those observed in swine subjected to 600 r. total body x-radiation.[12] The development of lesions in lymphoid organs and the alimentary tract was closely parallel in the two series. It is noteworthy that lesions in these two organ systems appeared earlier than elsewhere in the body. There was a slight time lag in the appearance of morphologic evidence of injury to the bone marrow, but once under way this injury progressed faster in the gamma-irradiated than in the x-irradiated swine.

Hemorrhagic lesions were seen only twice in the 7-day observation period in x-irradiated swine; but after 6 days in the gamma-irradiated swine they were found routinely in a widespread distribution.

The first spontaneous death in the present series occurred at the end of day 6, whereas there were no spontaneous deaths in the x-irradiated swine over the entire 7-day period. From lethal-dose studies[13] it is known that deaths in x-irradiated swine occurred on days 11 and 12 after 600 r. exposure, while in this report the spontaneous deaths of the gamma-irradiated swine, receiving about 700 r., occurred in the seventh day. It therefore appears justifiable to state that the gamma-irradiated swine received a more damaging exposure than the x-irradiated swine. Whether this was because of the larger dose received (700 r. vs. 600 r.), a more effective dose distribution through the animal body, a faster dose rate, a higher energy of radiation, the stress associated with prolonged confinement in the cylinders, the smaller

and more immature swine used, or a combination of these factors cannot be determined at this time. The higher energy of the radiation with the smaller animals used would favor a greater and more uniform depth dose in the gamma-irradiated swine. Because the two studies are not strictly comparable it might be worth while repeating the laboratory x-radiation experiment simulating, in so far as possible, the physical factors experienced in the field.

Mechanisms Leading to Death

At the dosage employed in this study the appearance of hemorrhages was followed closely by death in all swine. This is in keeping with previous studies.[12] The cause of the hemorrhage is probably a combination of factors. There is widespread disappearance of the hematopoietic elements in the marrow. Megakaryocytes are rare after a few days and from other observations a thrombocytopenia may be assumed.[14] There is congestion and dilatation of small blood vessels. Increased capillary permeability or fragility may be suspected.

Bacterial invasion and growth were widely evident in every animal dying spontaneously in this study. The necropsies frequently were done within minutes after such spontaneous deaths. The rôle of bacterial intoxication cannot be determined by this study, but that it may be a major causal factor of the commonly noted acute collapse and death cannot be denied. This bacterial invasion also may contribute to the development of hemorrhages.

Resistance to bacterial invasion is related to phagocytic as well as to humoral mechanisms. Efforts to evaluate the influence of irradiation on the phagocytic function of the reticulo-endothelial system have yielded varied results.[9,15-18] Tullis and his associates[17] observed no alteration of phagocytosis in rabbits at intervals ranging from 3 hours to 30 days following irradiation with doses of 500 and 800 r. The rate of disappearance of intravenously injected colloidal gold of 0.025 to 0.031 μ particle size was estimated in these studies. In more recent investigations with rabbits using the colloidal dye prodigiosin with a larger particle size (0.05 to 0.1 μ), Taplin and colleagues[18] found some impairment of phagocytosis 7 and 14 days after 800 r. total body radiation. Larger radiation doses of 1,000 and 1,200 r. appeared to produce a greater degree of impairment of phagocytosis after the same intervals. This latter observation may be contrasted with the morphologic demonstration of rapid phagocytic removal of cellular débris during the 24 hours immediately following irradiation as seen in this and other serial sacrifice studies.[12]

In evaluating radiation resistance it appears that the state of activi-

ty of the reticulo-endothelial system of the experimental animal may have to be considered.[18] Both the control x-irradiated swine[12] and the gamma-irradiated swine of this report were exposed to the same program of immunization by vaccines containing foreign protein. They would appear to represent like groups as far as activity of the reticulo-endothelial system is concerned.

Summary and Conclusions

The several lymphoid organs in a swine reacted in a roughly similar manner to an amount of total body gamma radiation which was lethal within 8 or 9 days for 100 per cent of the swine exposed. Necrosis of lymphoid cells was found well established by 4 hours after irradiation, but was even more extensive at 8 hours. There was an arrest of mitotic activity in the swine studied at 4 hours with a partial resumption of activity after 8 hours. The normal mitotic level was not regained but rather, after the first day, there was again a decrease but not cessation of activity. After a quiescent period, beginning repopulation of lymphoid organs with lymphoid cells was noted on day 3. Regeneration did not proceed far before death of the swine. Large mononuclear phagocytes were active in areas of lymphoid destruction during the first 24 hours, but not in the preterminal period.

Hematopoietic cells of the marrow began to diminish by 8 hours. These decreased steadily in number without evidence of regeneration before death. The erythroblasts were more sensitive than the myeloblasts, which in turn were more sensitive than the megakaryocytes. Terminally, the marrow was depleted of nearly all hematopoietic cells except the primitive reticular cells.

Early damage to the alimentary tract was limited to the epithelium lining the basal mucosal glands of the small intestine and to the lymphoid elements wherever these were present. This epithelium was rapidly repaired, being complete by the end of day 1, and plasma cells tended to replace many of the lymphocytes in the lamina propria.

In the testes the radiation injury studied here was limited almost entirely to destruction of spermatogonia.

The late stage of injury was characterized by multiple hemorrhages throughout the body, both into the tissues and into the lumina of hollow viscera. Ulcers in the mucosa of the alimentary tract were also observed frequently.

In pigs dying spontaneously, terminal invasion and growth of bacteria were prominent features. Pulmonary edema was a complicating factor.

No sex difference in reaction was apparent.

The more rapid development of lesions and shorter course of the disease with fatal termination observed in the gamma-irradiated swine here reported suggested that the "radiation effectiveness" was greater than in the previous studies of x-irradiated swine.

The type and sequence of organ and tissue injuries in the swine exposed to the gamma radiations from an atomic bomb under the conditions described herein were similar to those following 600 r. total body x-irradiation from a 2,000 KVP source as previously described.

We gratefully acknowledge the assistance given by Robert Veenstra, veterinarian; Clarence Lushbaugh, pathologist, and his staff at the Los Alamos Scientific Laboratory, where the tissues were blocked, cut, and stained; George H. Austin, photographer; and James S. Otto, Lloyd H. Dedon, and Donald J. Skelly, laboratory and necropsy technicians.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Illustrations of the gross pathology of which the legends are marked with an asterisk were taken from swine which received either more or less than 700 r. radiation in connection with a companion experiment.[13] Those chosen were indistinguishable from others of the 700 r. exposure group.

FIG. 1. Agranulocytic ulcers in the buccal mucosa of swine 447 which died 8 days after 845 r. irradiation.*

FIG. 2. Hemorrhage in the tonsils and oro-pharyngeal mucosa of swine 364 which died 7 days after 700 r. irradiation.

FIG. 3. Subpleural and intramuscular hemorrhages in swine 378 which died 11 days after 320 r. irradiation.*

FIG. 4. Marked edema and scattered hemorrhages in the lungs of swine 384 which died 6 days after 700 r. irradiation.

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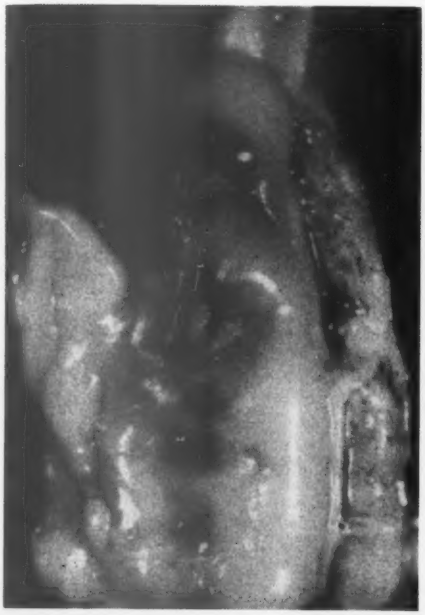

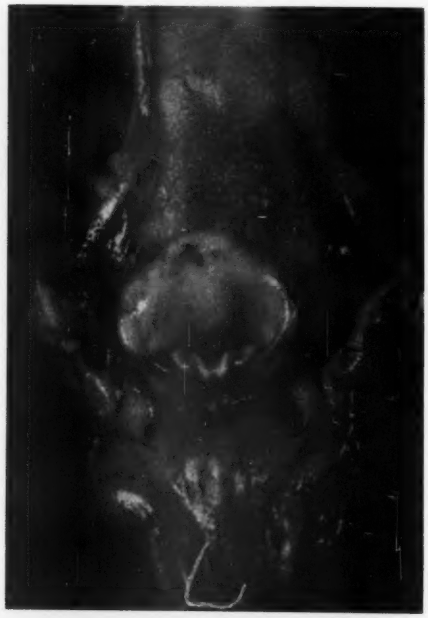
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FIG. 5. Hemorrhages in the parenchyma and pelvis of the kidney of swine 359 which died 7 days after 700 r. irradiation.*

FIG. 6. Hemarthrosis (ischiofemoral joint) in swine 357 which died 4 days after 1,000 r. irradiation.*

FIG. 7. Hemorrhage in the gastric and duodenal mucosa with sloughing of the superficial epithelium in the stomach of swine 364 which died 7 days after 700 r. irradiation.

FIG. 8. Multiple hemorrhages in the intestinal mucosa. The duodenum above is followed in order by the proximal small intestine, the distal small intestine, and the large intestine. Swine 412 died 9 days after 390 r. irradiation.*

FIG. 9. Hemorrhages in the bowel wall and mesenteric lymph nodes, intussusception, and fibrinous peritonitis in the small intestine removed en masse with attached mesentery from swine 359 which died 7 days after 700 r. irradiation.*

FIG. 10. Hemorrhagic edematous mucosa of the large intestine from swine 425 which died 6½ days after 700 r. irradiation.

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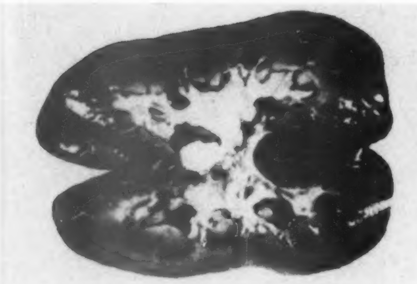

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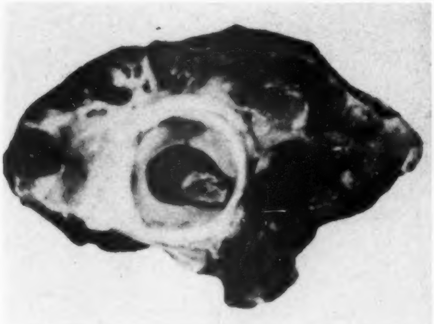

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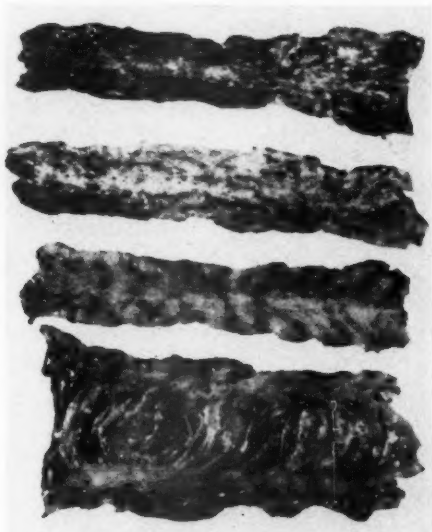

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FIGS. 11 to 18. Spleens from swine at serial times after exposure to about 700 r. total body gamma irradiation. × 250. *Fig. 11.* Swine 309, normal control. *Fig. 12.* Swine 418, 4 hours after irradiation. *Fig. 13.* Swine 475, 7½ hours. *Fig. 14.* Swine 428, 25 hours. *Fig. 15.* Swine 327, 2 days. *Fig. 16.* Swine 330, 5 days. *Fig. 17.* Swine 384, 6 days. *Fig. 18.* Swine 364, 7 days. The débris from lymphocyte destruction is evident at 4 hours, increased at about 8 hours, but almost entirely removed by phagocytosis at 1 day. There is an abortive attempt at repopulation of the malpighian corpuscles with lymphocytes over the next few days, but as spontaneous deaths begin to occur after 6 days the number of lymphocytes decreases again and hemorrhages appear. Bacterial colonies (arrows) are present in the animals which died spontaneously (Figs. 17 and 18). There is no inflammatory response to the bacterial invasion.

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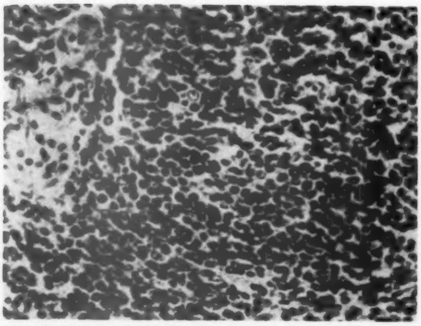

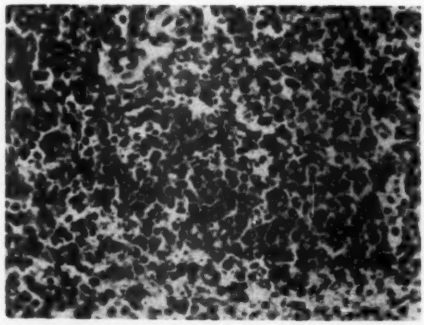
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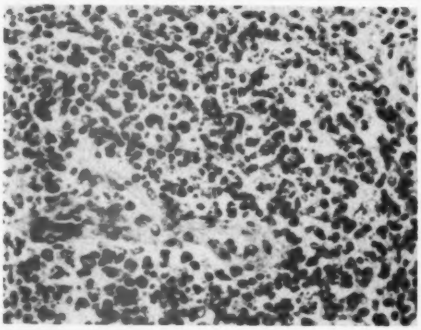

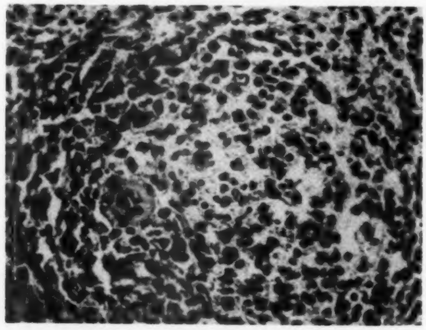
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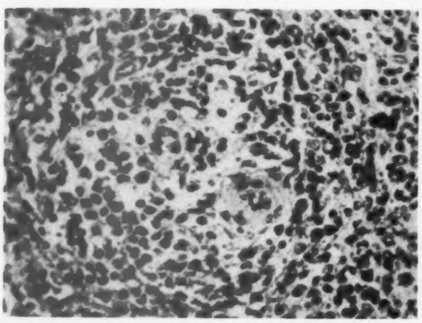

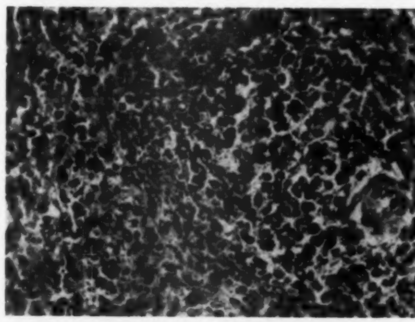
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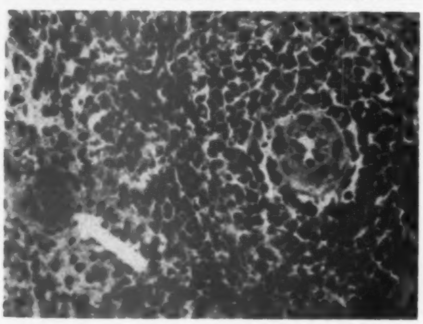

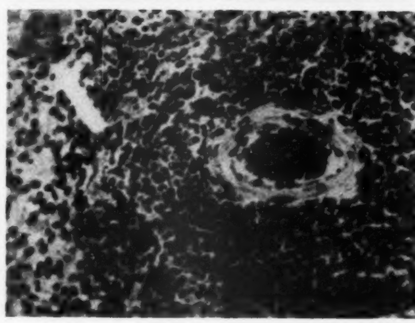
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FIGS. 19 to 26. Sternal marrow from swine at serial times after exposure to about 700 r. total body gamma irradiation. × 250. *Fig. 19.* Swine 477, 4 hours after irradiation. *Fig. 20.* Swine 475, 7½ hours. *Fig. 21.* Swine 340, 15½ hours. *Fig. 22.* Swine 472, 2 days. *Fig. 23.* Swine 462, 3 days. *Fig. 24.* Swine 352, 4 days. *Fig. 25.* Swine 393, 6 days. *Fig. 26.* Swine 364, 7 days. The hematopoietic cells steadily decrease, beginning about 8 hours after irradiation. The blast cells are destroyed first. The nuclei of the megakaryocytes become pyknotic early, but these cells do not decrease in numbers until the third or fourth day. The adult blood cells and the reticular cells are the last to disappear.

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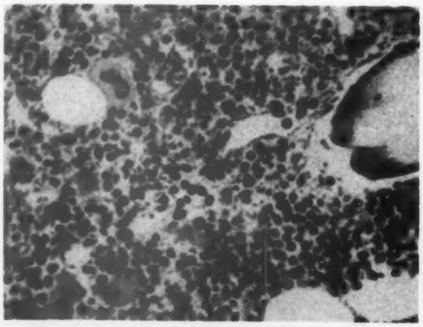

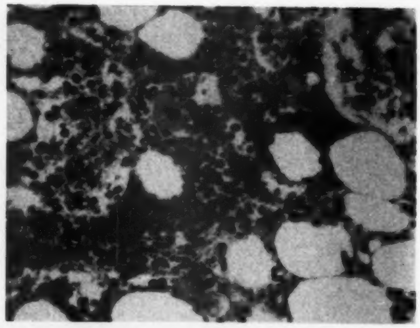
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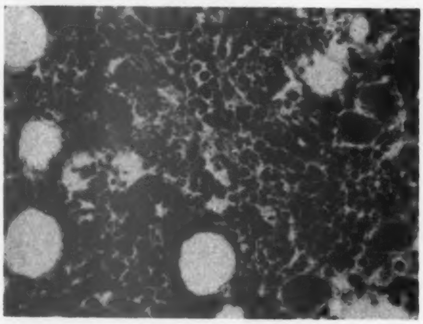

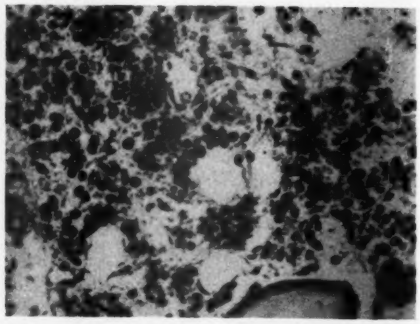
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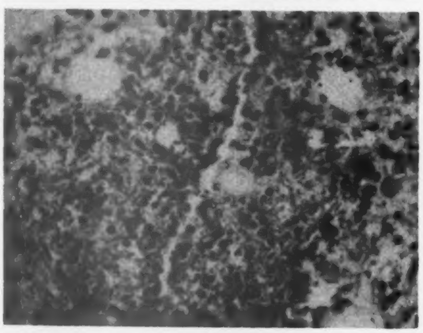

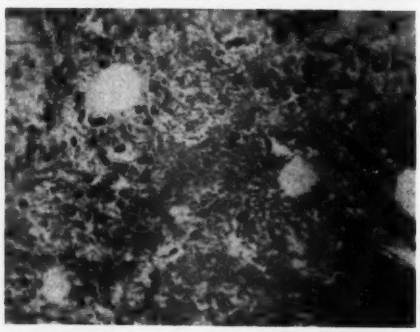
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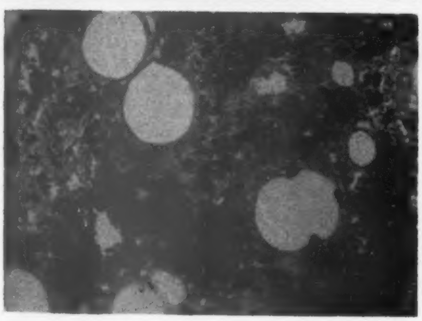

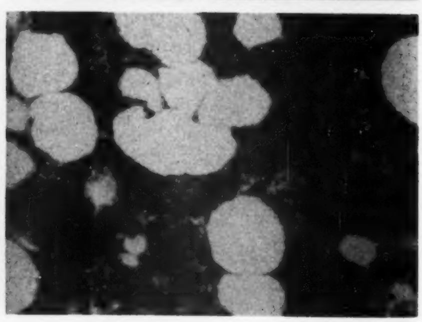
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FIGS. 27 to 30. Small intestinal mucosa from swine at serial times after exposure to about 700 r. total body gamma irradiation. × 475 except Fig. 30 which is × 125. *Fig. 27.* Swine 477, 4 hours after irradiation. *Fig. 28.* Swine 430, 7½ hours. *Fig. 29.* Swine 338, 25 hours. *Fig. 30.* Swine 405, 6 days. The glandular epithelium which is injured early is rapidly replaced. The early inflammatory reaction also disappears within 1 day. Lymphocytes in the lamina propria become decreased and plasma cells become more numerous. A day or two prior to death the small blood vessels become dilated and hemorrhages in the mucosa are frequent. Erosions and ulcerations are natural sequelae to the hemorrhages.

FIG. 31. Lungs from swine 393, 6 days after 700 r. irradiation. The acellular fibrinoserous exudate, vascular congestion, and bacterial colonies (arrows) are characteristic of the lungs of the swine which died spontaneously. × 250.

FIG. 32. Liver from swine 384, 6 days after 700 r. irradiation. The parenchymal cells appear normal, but a bacterial colony (arrow) is present. × 250.

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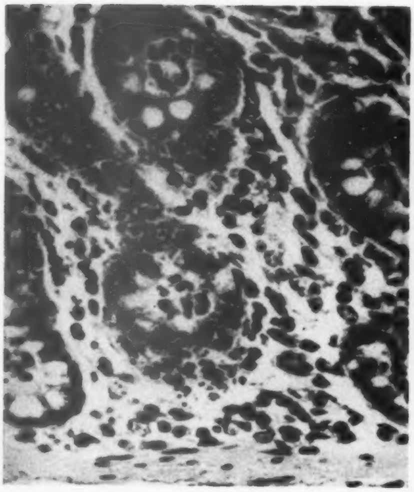

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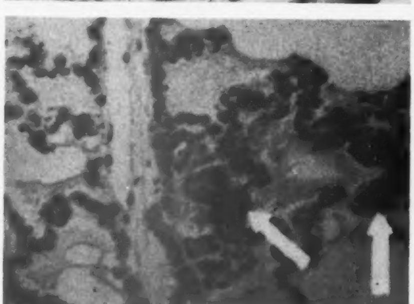

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HETEROTOPIC OSSIFICATION IN INTESTINAL NEOPLASMS *

H. Terry Van Patter, M.D.,† and J. W. Whittick, M.B.
From the Department of Pathology, Royal Cancer Hospital, Fulham Road, London, S. W. 3, England

The connective tissue stroma of epithelial neoplasms is a relatively uncommon site of heterotopic ossification. Previous reports have described its occurrence in benign and malignant tumors of widespread origin, including adenocarcinomas of the rectum, colon, appendix, ileum, stomach, gallbladder, uterus, breast, prostate and salivary glands, calcified "epitheliomas" of the skin, sweat gland adenomas, bronchial adenomas, and craniopharyngiomas.[1] Of these, intestinal adenocarcinomas in general, and rectal adenocarcinomas in particular, account for the majority of cases, although even in the latter group the incidence is extremely low, having been estimated at 0.4 per cent.[2]

Despite the interest in heterotopic ossification, as evinced by the abundant literature, its precise morphogenesis has remained obscure. Examination of 3 hitherto unreported cases of adenocarcinoma of the large intestine with stromal ossification has revealed certain features which appear to contribute to an understanding of the process.

Report of Cases

Case 1

F. N. was a white male butcher, 43 years old, who first noticed blood in the stools in July, 1947. Exploratory laparotomy at another hospital established a diagnosis of carcinoma of the rectum and colostomy was performed. He was then referred to the Royal Cancer Hospital for definitive therapy. Here biopsy confirmed the diagnosis and an abdominoperineal excision of the rectum was done in September, 1947, at which time no metastases were evident. Gross examination of the surgical specimen disclosed in the distal rectum a large, fungating tumor measuring 6.0 cm. in diameter, which penetrated through the wall of the bowel. The postoperative course was stormy, being complicated by intestinal obstruction which necessitated laparotomy and by subsequent development of a perineal fecal fistula. Eventually the patient made a satisfactory recovery and was discharged in good health in December, 1947. He remained well and at work until February, 1952, when he complained of cough and hemoptysis. Multiple bilateral pulmonary opacities, considered to be metastases, were found in radiographs of the chest. Cytologic examination of aspirated pleural fluid confirmed the presence of malignant cells. Symptomatic therapy was instituted and three courses of an experimental chemotherapeutic agent were administered with some subjective improvement. The pulmonary metastases gradually increased in size and caused marked dyspnea. His general condition deteriorated and he died in this hospital in August, 1953, 6 years after the onset of rectal bleeding and 1½ years after the recognition of pulmonary metastases.

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† Allan Blair Memorial Fellow, Canadian Cancer Society. Now at: Department of Pathology, McGill University, Montreal, Que.

Gross Findings

Necropsy was performed by one of us (J.W.W.) 80 hours after death. Examination of the thorax revealed complete obliteration of the right pleural cavity and partial obliteration of the left by firm adhesions; the patent portion of the latter contained blood-stained liquid. The parenchyma of both lungs was extensively replaced by necrotic, gelatinous, and partly gritty neoplastic tissue. The small gritty areas consisted of opaque, yellowish white, granular material mixed with minute, irregular, bony-hard, whitish spicules embedded in a translucent, gray, mucinous matrix. Gross permeation of large bronchi and blood vessels by tumor was visible in the right lower lobe and left upper lobe. The scanty non-involved lung tissue was poorly aerated due to multiple irregular areas of edema, pneumonic consolidation, and abscess formation. Copious thick, yellow, mucopurulent material was present in the trachea and bronchi. The hilar and other mediastinal lymph nodes were enlarged but showed no gross involvement by tumor. The thoracic duct and cisterna chyli were normal. There was bilateral slight enlargement and induration of axillary lymph nodes.

The pericardial sac was not invaded by tumor but contained a 100 cc. effusion of clear, straw-colored liquid. The heart weight was within normal limits and there was no gross cardiac abnormality. Minimal atherosclerosis was present in the coronary arteries and aorta.

In the abdomen were a few fibrous peritoneal adhesions but no tumor deposits. The liver, spleen, and kidneys, though slightly increased in weight due to passive congestion, were tumor-free. The pancreas, adrenal glands, and genito-urinary tract were normal. The esophagus, stomach, and intestine, which terminated in a patent colostomy, showed no lesions. The soft tissues of the pelvis and perineum revealed no residual tumor and the pelvic and abdominal lymph nodes were normal in size and consistency. Two right inguinal lymph nodes were slightly enlarged and indurated.

Within the right cerebral hemisphere were two discrete tumor nodules, the larger measuring 4.3 cm. in greatest diameter. They were soft and gelatinous throughout and contained no gritty areas. The pituitary gland appeared normal.

The remainder of the examination of the head and neck showed a normal thyroid gland and small, soft, cervical lymph nodes. The upper respiratory and alimentary passages were not remarkable.

There was no evidence of tumor growth in the skull, ribs, or lumbar

vertebral bodies. The skin and extremities showed no abnormality and the musculature was well developed.

Microscopic Findings

Sections of the surgical specimen showed a moderately well differentiated adenocarcinoma with focal mucinous areas. It had penetrated through the muscular wall of the rectum, but neither blood vessel invasion nor lymph node metastasis was present. There was no calcification or ossification.

Multiple sections of the extensive metastatic tumor deposits found in the lungs at necropsy disclosed numerous clumps of moderately well differentiated adenocarcinoma cells which lay in and around large pools of mucin and necrotic débris (Fig. 1). Tissue breakdown was indicated by the presence of nuclear débris, cholesterol clefts, and collections of foamy macrophages (Fig. 2). The malignant cells were identical in appearance with those of the primary rectal tumor. There was, however, considerably more mucin production and necrosis, so that there were large areas devoid of viable cells. Calcium deposition within such areas was extensive, varying from small, scattered, deeply basophilic granules to clumps and extensive irregular plaques (Fig. 3). Traversing many of the pools of mucin was a fine to coarse connective tissue network which contained proliferating fibroblastic cells and occasional capillaries but with a notable absence of inflammatory cells (Fig. 4). These fibrous trabeculae appeared to broaden with maturity and many had become densely fibrous and hyalinized, often incorporating calcified plaques.

Superimposed on this background of organized and calcified mucinous tumor secretion were multiple scattered foci of intramembranous ossification. The heterotopic bone seemed to be formed in three slightly differing morphologic situations.

First, it appeared most commonly in apposition to masses of calcium, where it developed by metaplasia within the surrounding fibroblastic connective tissue (Figs. 5, 6, and 7). Numerous transitions were readily traced which demonstrated the rounding-up of the fibroblasts to osteoblasts and the acidophilic homogenization of the collagenous intercellular matrix to form osteoid (Figs. 5, 6, and 8). The connective tissue involved in this change tended to be immature and many of the cells in areas of osteoid formation retained a basophilic mantle of mucin. This produced a chondroid appearance in some areas (Figs. 3 and 5), but no true cartilage or evidence of endochondral bone

formation was recognized. The deposition of calcium salts and the incorporation of the osteoblasts as osteocytes advanced the process and the resultant new bony trabeculae, haphazardly arranged about the calcific deposits, were easily recognized. As a secondary change, the connective tissue between some of the trabeculae appeared loosened and myxomatous, and in it developed delicate thin-walled sinusoidal blood vessels (Fig. 7). The addition of a light infiltrate of histiocytes, lymphoid and plasma cells, simulating rudimentary hemopoietic tissue, completed the resemblance to cancellous bone. No haversian systems were detected, but the presence of occasional groups of osteoclasts indicated resorptive activity.

Second, small spicules of bone were seen in the fibrous trabeculae. These had doubtless been formed in a manner similar to that described in the preceding paragraph but were now not associated with calcium deposits (Fig. 8).

Third, many of the large, irregular, calcified plaques which lay in the mucinous lakes or were surrounded by fibrous tissue appeared to have incorporated a few connective tissue cells and then to have undergone direct transformation into poorly formed coarse bone, without the intervening appearance of osteoblasts or osteoid (Fig. 9). This type of ossification was most prominent in areas with excessive calcium deposition, and, at the same time, a paucity of proliferating fibroblastic tissue.

The relationship of viable tumor cells to the foci of ossification was inconstant, as can be seen in the series of photomicrographs. In a few places the bony trabeculae were aligned with their long axes parallel to adjacent rows of tumor cells (Fig. 8), but, in the main, they appeared to have a more frequent localization in the immediate neighborhood of calcium deposits (Figs. 5, 6, and 7). In addition, it was noted that the zones of osteoid and bone formation occurred in relatively avascular areas of the proliferating fibroblastic connective tissue, as was indicated by their distance from the accompanying blood capillaries. Due, undoubtedly, to progressive tumor growth and further circulatory changes, many of the bony trabeculae and surrounding fibrous tissue trabeculae had become secondarily necrotic.

Large bronchi were invaded and occluded by neoplastic tissue. Adjacent branches of the pulmonary arteries showed marked intimal fibrous thickening.

In the surrounding lung parenchyma there were areas of bronchopneumonia, with formation of small abscesses, and also diffuse chronic

inflammatory changes consistent with long-standing bronchial obstruction. The visceral pleura was the seat of marked fibrous thickening. A tracheobronchial lymph node contained microscopic deposits of metastatic tumor in which there was no calcification or ossification.

The cerebral metastases revealed both abundant formation of mucin and large zones of necrosis. As in the lung, the pools of mucinous and necrotic material showed early organization by a proliferative fibroblastic network but there was neither calcification in the mucin nor ossification in the immature fibrous stroma.

No significant histologic changes were found in the liver, kidneys, pituitary gland, or axillary lymph nodes.

Case 2

G. H., a white seaman, 46 years of age, had a partial left colectomy for carcinoma in December, 1948, at Capetown, South Africa. In August, 1950, a small nodule in the vicinity of the umbilicus was excised at another hospital and proved to be a secondary deposit of adenocarcinoma. He was first seen at the Royal Cancer Hospital in April, 1951, when he complained of a nodule in the operative scar below the umbilicus and of post-prandial abdominal pain, distention, nausea, and of being easily fatigued. The nodule was widely excised and exploration of the abdomen failed to disclose additional metastases. A barium enema and chest roentgenograms were non-contributory. The gross examination of the surgical specimen revealed a mass of grayish white neoplastic tissue, measuring 3.0 cm. in greatest diameter, involving skeletal muscle but not the overlying skin or subcutaneous tissue. He was discharged in good health to be followed as an out-patient. In February, 1954, he was well and apparently free of recurrent growth.

Microscopic Findings

Sections of the nodule showed infiltration of skeletal muscle and fascia by moderately well differentiated, mucin-secreting adenocarcinoma of intestinal type containing necrotic foci. Small pools of mucin contained granular calcific deposits and were frequently invaded by strands of proliferating fibroblasts from the surrounding abundant dense fibrous stroma. Bony metaplasia in the connective tissue in these areas (Fig. 10) showed nicely the transition phases through osteoblastic differentiation and osteoid formation to bony trabeculae as described in case 1. The location of the bony spicules demonstrated no specific relationship to either tumor epithelium or the calcific deposits, but tended to occur in relatively avascular areas devoid of inflammatory change. A section of the primary tumor was reviewed and no bone formation was found. The first recurrence in the abdominal wall, however, showed areas of mucin, calcification, and ossification, the bone having similar relationship to that in case 1.

Case 3

A. B., a white male, 58 years old, developed rectal bleeding, and, at laparotomy in June, 1951, at another hospital, was found to have an inoperable carcinoma of the sigmoid colon associated with a pelvic abscess. A colostomy was performed in August, 1951. His general condition remained good for over a year and for this reason he was referred to Mr. A. Lawrence Abel for consideration of further therapy. He was admitted to the Princess Beatrice Hospital in June, 1952, where physical examination and a barium enema confirmed the diagnosis. An abdominoperineal resection of the rectum was carried out in conjunction with removal of a loop of adherent small bowel and the colostomy was revised. Multiple hepatic metastases were found at operation. The surgical specimen, examined at the Royal Cancer Hospital, disclosed to the naked eye an ulcerated, constricting tumor encircling the rectum at the line of peritoneal reflection and penetrating through its wall to invade an adherent loop of small intestine to the depth of its submucosa. No lymph node metastases were found.

Postoperative recovery was satisfactory except for persistence of a perineal sinus. This was scraped in June, 1953, and histologic examination of the material removed showed inflammatory tissue with no evidence of tumor. In February, 1954, his condition was fair with occasional bouts of upper abdominal pain but without weight loss.

Microscopic Findings

A section through the base of the carcinomatous ulcer in the rectum revealed a moderately well differentiated, partly mucinous adenocarcinoma which penetrated through the muscular wall and invaded the serosal aspect of the adherent loop of small intestine. Considerable necrosis of tumor was present and small pools of secreted mucin lay in a dense fibrous stroma. Small deposits of calcium were seen frequently within the necrotic débris and pools of mucin (Fig. 11). Little tendency to fibroblastic organization was apparent, and no ossification was evident at this site. However, in the portion of tumor invading the small bowel there were numerous bony spicules within the proliferating connective tissue stroma and lying between columns of tumor cells (Fig. 12). In contrast to cases 1 and 2, the ossification in this location did not appear in zones of mucinous infiltration nor in close relationship to calcific deposits, although, as mentioned, both of these features were noted nearby.

Discussion

A brief consideration of the histogenesis of heterotopic ossification in general seems pertinent. Leriche and Policard[3] discussed it at considerable length in their monograph on the pathophysiology of bone. They stated that the essential features of this process are the existence of an ossifiable connective tissue medium and the presence of a calcific deposit in the vicinity. The ossifiable medium is described as a connective tissue which, because of inadequate blood supply, has become

edematous and has reverted to an "embryonic" state. This involves swelling and multiplication of elementary collagenous fibrils followed by infiltration with "pre-osseous substance" (osteoid). The simultaneous resorption of nearby organically fixed calcium salts produces a "local calcific surcharge" whereupon ossification takes place by the usual processes.

Their interpretation is strongly supported by the structural features of the majority of the more common instances of bony metaplasia in fibrous tissue surrounding non-neoplastic lesions which contain calcified necrotic or degenerate tissue, such as caseous tuberculous foci, atheromatous plaques, and thyroid nodules. It is also supported by the finding that the bone resulting from heterotopic ossification contains calcium and phosphorus in the same proportion as does normal skeletal bone.[4]

Contrary opinions are, however, also based on considerable pathologic and experimental evidence, as Willis[1] has pointed out in his admirable monograph on the subject of metaplasia. For instance, in many examples of heterotopic ossification as seen in laparotomy scars and myositis ossificans, no local calcific deposits are demonstrable by the usual histologic methods. Furthermore, experimentally transplanted epithelium of the urinary bladder, proliferating in contact with connective tissue in certain situations, induces in it bony metaplasia without associated calcification.[5,6] The transplantation of gallbladder and gastric mucosa has produced similar results,[7] but the mechanism of this inductive effect is far from clear.[8] In addition, the experimental introduction of calcium in various forms into the tissues of laboratory animals in an attempt to induce osteogenesis has led to conflicting results.[3,6,8] Apart from the recognition that zones of osteoblastic activity are constantly rich in alkaline phosphatase,[8,9] enzyme studies have not clarified the problem. Thus it appears that the morphologic criteria enumerated by Leriche and Policard[3] as essential to heterotopic ossification are present frequently but not constantly.

Although differing over the nature of the stimulus to osteogenesis, most recent writers have agreed that it is manifested through metaplasia *in situ* of the pre-existing fibroblastic connective tissue.[1,3] Keith,[10] however, proposed that the earliest osteoblastic cells were derived from proliferating vascular endothelium. The occasional subsequent development of bone marrow containing hemopoietic elements affirms the validity of the concept of the pluripotentiality of proliferating mesenchymal tissues.[1]

As to the more specific problem of heterotopic ossification in the

stroma of intestinal adenocarcinomas, published reports fail to give a clear account of its morphogenesis. Most of the histologic features described in our 3 cases have been mentioned by previous authors, but none has noted the striking appearance of massive calcification in the tumor-secreted mucin shown in case 1, and to a lesser extent in cases 2 and 3. In an attempt to interpret the over-all importance of this feature as a stimulus or localizing factor in the osteogenesis under consideration, we have re-examined the histologic material from the 3 cases previously reported from this hospital by Christie,[11] and 2 other cases with bone formation in metastases of rectal adenocarcinomas referred here for consultation. In none of these cases were significant calcific deposits demonstrable in the material available for study, although necrosis and mucin production were seen in varying degree. Two cases of transitional cell carcinoma of urinary tract epithelium, containing metaplastic stromal bone, one from the bladder and the other from the pelvis "in the position of the urachus," were studied. Neither contained mucin but one case revealed extensive necrosis with focal areas of calcification. Finally, a case of recurrent malignant "mixed" tumor of the parotid gland showed metaplastic ossification of its stroma without obvious necrosis, calcification, or mucin production. To complete the picture, examination was made of the material from several cases of rectal adenocarcinoma, including both primary and secondary lesions, in which there was extensive calcification, but no bone formation. In the main, the calcium salts were deposited in areas of coagulative necrosis of tumor tissue rather than in secreted mucin, but in one section an occasional focus of calcification was found in a pool of mucin surrounded by proliferating fibroblastic tissue. Thus the stage would seem to have been set for bone formation. To explain its absence, we can only assume that the local circulatory status was not conducive to ossification or that a time factor is involved, insufficient time having elapsed for bone to have appeared.

It appears clear from the study of this additional material that the presence of calcification in mucin is by no means a constant feature of the development of bone in the stroma of epithelial neoplasms, although obviously an important one in the 3 cases which have been described. Conversely, as might be expected, its presence is not inevitably associated with bone formation as far as can be determined from the material examined. In the case of recurrent rectal adenocarcinoma reported by Senturia, Schechter, and Hulbert,[12] heterotopic bone predominated in areas of necrosis and mucinous degeneration,

and in some of these areas scattered calcium granules were observed. Although their illustrations do not demonstrate recognizable calcification in mucin, it appears likely that the morphologic features were essentially similar to our 3 cases. A closer resemblance obtains in the case of a mucocele of the appendix reported by Juvara and Borcescu[7] in which extensive ossification appeared in areas of calcification and necrosis separated by granulation tissue from islands of epithelium in the presence of mucoid impregnation of the whole structure. Christeller and Mayer[13] illustrated a similar case in which bone formation in the wall of an appendicular mucocele was associated with calcification and mucinous infiltration. However, the majority of writers[1,2,11,14,15] have failed to find calcium deposits in their material and have, therefore, tended to reject the previously mentioned theory of histogenesis advanced by Leriche and Policard[8] which requires among its essential factors the presence of a local calcium deposit.

The hypothesis most frequently offered in its place postulates the existence of a specific stimulus to stromal osseous metaplasia derived from some property peculiar to the epithelium of the particular tumor and presumably related to enzymes or metabolites produced by the tumor cells.[1,11,14,15] We are not aware of the application of any histochemical studies which support or deny this hypothesis, although the final step-by-step elucidation of the whole problem probably lies in that field of investigation. Thus, while a categorical denial of the existence of such an epithelial stimulus is not called for, it is equally clear that the validity of this concept remains to be established, and, at the same time, there are a number of anatomical facts which challenge it. First, no one has been able to detect any histologic difference between the malignant cells of tumors which form bone in their stroma and those of the (commoner) tumors which do not form bone. Second, the cases we have described show that in at least some instances the presence of calcification adjacent to proliferating immature connective tissue is associated with heterotopic bone formation. Third, in the cases recorded, the occasional occurrence of bone formation in recurrent or metastatic lesions, with no evidence of its presence in the primary tumor, seems difficult to understand on the suggested basis of a specific epithelial stimulus, as also is, fourth, the unexpectedly frequent localization of these secondary lesions in the anterior abdominal wall. Fifth, the well known tendency of necrotic tissue to undergo calcification lends support to the impression that diligent examination of bone-containing adenocarcinomas, almost all of which will disclose necrosis to some degree, might reveal the presence of calcium deposi-

tion more often than has been noted in the literature. Indeed, as Leriche and Policard[8] have stated, "If one takes into account the frequency of zones of necrosis in these new growths, one need not be astonished that there can be points of calcification in them, and the latter are probably the origin of the bone plates found in such tumours."

On the basis of our 3 cases, the additional material examined, and the cases recorded in the literature, we would prefer to take the middle road and support a histogenetic theory based on the osseous metaplasia of proliferating mesenchymal tissue which is stimulated by the interaction of local physicochemical factors. The deposition of calcium salts in accumulations of mucin represents one such factor to be considered of importance in the cases in which it is present. In those cases in which apparently it is absent, we do not agree that it is necessary to postulate the existence of a peculiar epithelial property which stimulates osteogenesis. It may rather be that some morphologically undetected factor is operating locally to produce a "calcific surcharge."

Necrosis has been suggested by some as playing a part.[2,16] However, as almost all adenocarcinomas of the intestine will show some necrosis, it is in itself unacceptable as a significant factor, although probably playing an indirect rôle by predisposing to calcification.

The secretion of mucin by tumor cells, usually in minimal amount, has been present in many of the cases reported. This can be recognized, however, in a large percentage of ordinary intestinal adenocarcinomas, particularly if special stains for mucin are carried out. Furthermore, in those with abundant mucin production, there does not appear to be an increased incidence of heterotopic bone formation. In our material mucin appears to play its part by providing an avascular medium susceptible to calcification and organization by embryonic connective tissue. This seems a more significant factor than necrosis. Whether the mucin may have any chemical contribution to the osteogenic process is at present conjectural.

An early observation on the rôle of local circulation in the formation of heterotopic bone is attributed[3,6] to von Recklinghausen who observed that slowing of the lymphatic current is a condition necessary for calcification. Leriche and Policard[8] noted that zones of preosseous edema and osteoid formation tend to appear in relatively avascular areas of the ossifiable connective tissue medium, as indicated by their distance from the accompanying blood capillaries. We found a fairly impressive relationship of this sort in cases 1 and 2, but it was difficult to confirm in the remainder of the material studied. Binkley and Stewart[17] also have considered that local tissue anoxia resulting

from stagnation of blood flow is a factor of prime importance in the development of calcification and ossification in a hyalinized connective tissue, but, as morphologic evidence for this viewpoint, they referred to the nearby presence of thin-walled cavernous vessels which, they believed, result from obstructive dilatation. Scheidegger[15] went so far as to call the heterotopic bone in his 2 cases of rectal adenocarcinoma "angiogenic" bone, because he observed its formation in the vicinity of blood vessels.

This discrepancy of opinion with regard to the relationship of heterotopic bone formation to blood vessels can be resolved if the appearance of the thin-walled sinusoidal vessels lying adjacent to and between well formed bony trabeculae is considered to be a secondary change; namely, a manifestation of the architectural reorganization of the newly formed bone and of its contained connective tissue. The formation of a distinct periosteal limiting layer about the trabeculae, lacunar osteoclastic resorption, and the appearance of fat cells and hemopoietic elements in the connective tissue spaces represent other secondary changes which may develop in the heterotopic bone.[1,3,11] These changes are analogous to those seen in the reorganization of newly formed skeletal bone. This similarity of biologic development reinforces the concept that the respective histogenetic mechanisms are also alike.

In his analysis of 4 cases of rectal adenocarcinoma containing metaplastic stromal bone, Dukes[2] suggested that a tendency to slow growth, as was indicated by the clinical course and the histologic appearances, represented a common feature. More recent reports,[11,12] however, have failed to confirm this contention, inasmuch as several of the cases have shown rapid growth with early and wide dissemination leading to death in 1 to 2 years following the onset of symptoms. Our case 1, with a total survival of 6 years, is probably best considered of long average duration, as is case 2 with a survival of over 5 years at the time of writing; whereas the follow-up in case 3 is too short for consideration. Our cases contribute little to this question, but we feel that no significant difference has been established in the biologic behavior of these bone-containing tumors as compared with intestinal adenocarcinomas in general.

Finally, it is interesting to note that in at least 3 of the recorded cases of intestinal adenocarcinoma containing bone, of which we have found a total of 19,[2,11,12,14-16,18-20] with inclusion of our 3, the bone has developed in the stroma of secondary tumor deposits within surgical scars in the anterior abdominal wall as described in Clark's case,[18]

Christie's[11] case 2, and our case 2. This frequency of osseous metaplasia in metastases in the abdominal wall seems disproportionately high and suggests that it may share a common factor with another well known instance of heterotopic ossification, namely, that occurring in laparotomy scars. Only one of our cases, case 2, falls into this group, and its histologic examination does not clarify the nature of this association. It might be thought possible that the tumor deposits in these 3 cases are simply metastases to heterotopic bone within incisional scars, but we consider it far more likely on morphologic grounds that the bone formation is similar in its origin to that occurring in the other tumors described. It may be that the peculiar susceptibility of the proliferating fascia of the anterior abdominal wall to undergo heterotopic ossification[6] represents an addition to the local factors responsible in these cases for osteogenesis.

Summary and Conclusions

Three cases of intestinal adenocarcinoma with heterotopic stromal ossification are reported. The histologic features are described and illustrated and the predominating morphologic features are discussed in relation to the mechanism of this bone formation and to its previous interpretations. It is concluded that calcium deposits play an important rôle in the induction and localization of metaplastic ossification in the stroma of some mucin-secreting intestinal adenocarcinomas.

Permission to use the clinical histories was kindly given by Mr. A. Lawrence Abel for cases 1 and 3 and by Mr. R. W. Raven for case 2. We are grateful to Mr. J. Heselson of Cape Town for sections of the first two surgical specimens of case 2. The histologic preparations and photomicrographs are the work of Mr. R. A. Outteridge, A.I.M.L.T.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

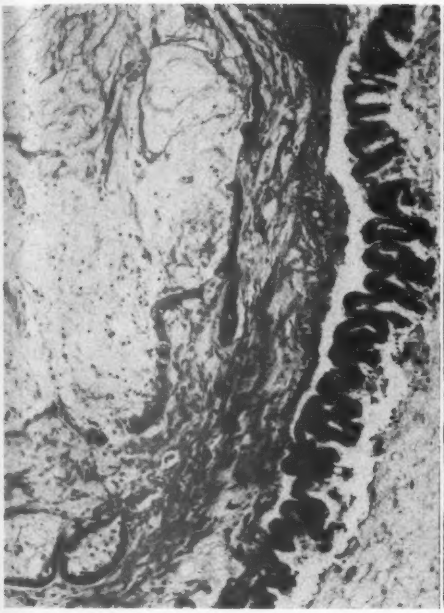
All sections illustrated were fixed in 10 per cent formalin and stained with hematoxylin and eosin.

FIG. 1. Case 1. Pulmonary metastasis. Early dissolution of tumor cells at periphery of pools of accumulated mucinous secretion. × 75.

FIG. 2. Case 1. Pulmonary metastasis. Necrosis and mucinous infiltration with proliferation of stroma from surviving nests of tumor. × 75.

FIG. 3. Case 1. Pulmonary metastasis. Calcium deposits in mucin. A few connective tissue cells incorporated at lower right resemble cartilage cells. × 125.

FIG. 4. Case 1. Pulmonary metastasis. Fibroblastic organization of mucin. × 75.



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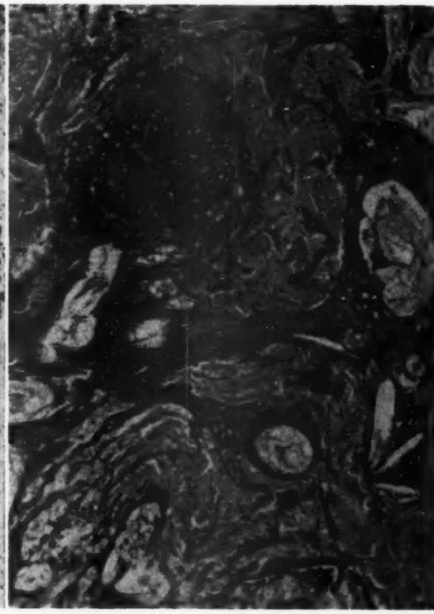

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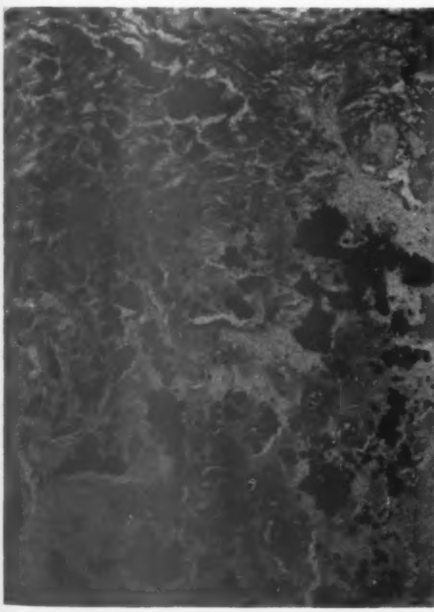

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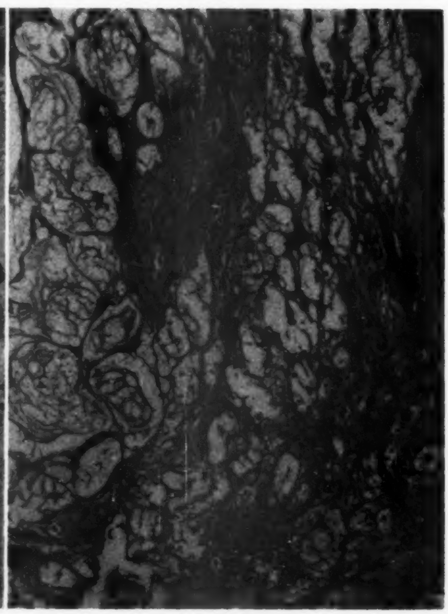

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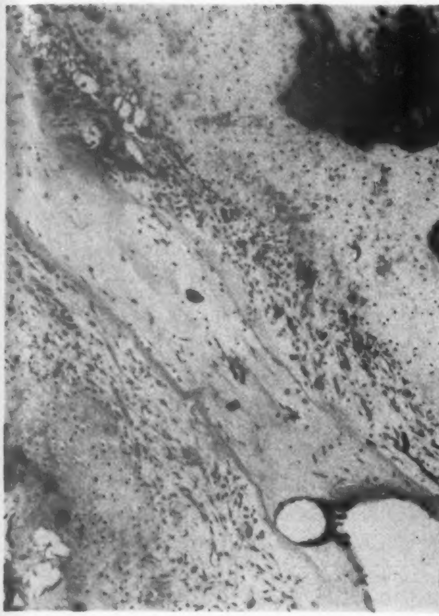
FIG. 5. Case 1. Pulmonary metastasis. Osteoid tissue at lower left and upper right where it surrounds a calcific plaque. A narrow rim of bone lies in apposition to the lower edge of the plaque. The faint stippling around some of the osteoblasts represents a residue of mucin and imparts a chondroid appearance. There is relative avascularity of zones of ossification compared with the non-ossifying connective tissue centrally. × 75.

FIG. 6. Case 1. Pulmonary metastasis. Metaplastic bone formation within proliferating fibroblastic connective tissue adjacent to large focus of calcification in pool of mucin below. Smaller accumulations of mucin in spaces above. × 75.

FIG. 7. Case 1. Pulmonary metastasis. Similar to Figure 6, showing bone formation about calcific deposits. There are large sinusoidal blood vessels lying in loose connective tissue between the bony trabeculae in the upper portion of the field. × 75.

FIG. 8. Case 1. Pulmonary metastasis. A calcified bony spicule lies parallel to a row of tumor cells on either side. Osteoid tissue above and below is surrounded by a layer of plump osteoblasts. × 75.



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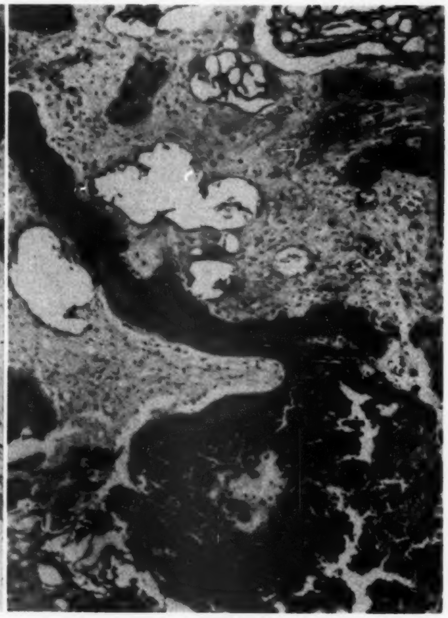

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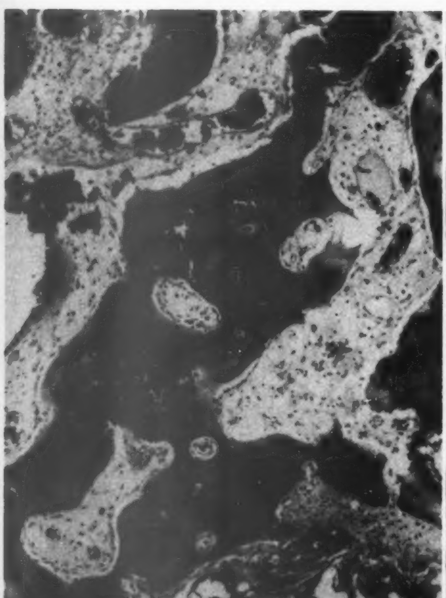

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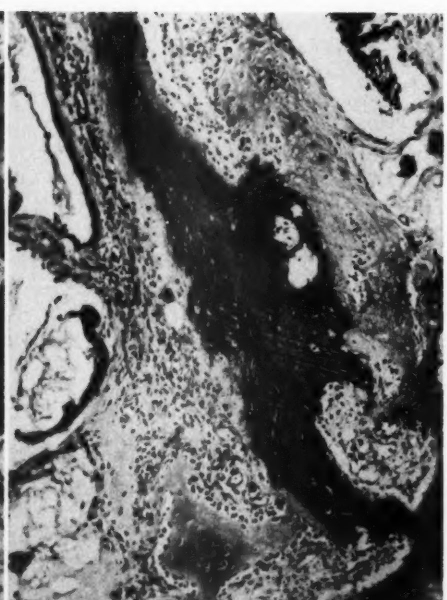

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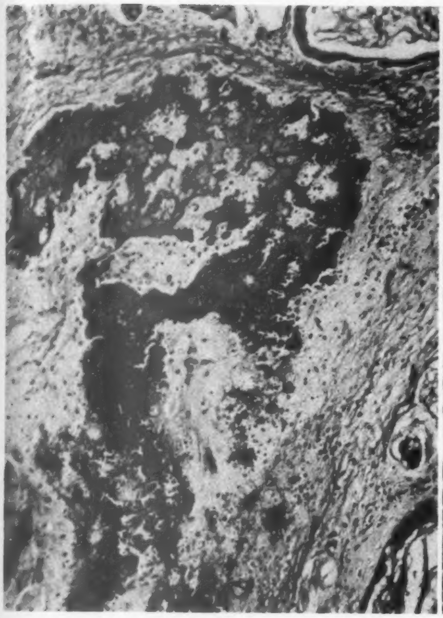
FIG. 9. Case 1. Pulmonary metastasis. Large calcific plaque, lying in partially organized mucinous matrix, has undergone in its upper portion direct transformation into imperfect bone by the incorporation of a few connective tissue cells. × 75.

FIG. 10. Case 2. Secondary adenocarcinoma in scar of abdominal wall. Large calcium deposit at upper left lies in mucinous secretion which is being infiltrated by proliferating fibroblasts. Two small bony spicules at lower center. × 125.

FIG. 11. Case 3. Primary adenocarcinoma of rectum. Nests of degenerative mucin-secreting tumor cells within dense fibrous stroma in ulcerated base of primary lesion. Small irregular calcium deposits are seen in mucin, necrotic tumor, and stroma. × 75.

FIG. 12. Case 3. Adenocarcinoma invading small bowel by direct extension from rectal primary. A group of small bony spicules lies in the tumor stroma. × 125.



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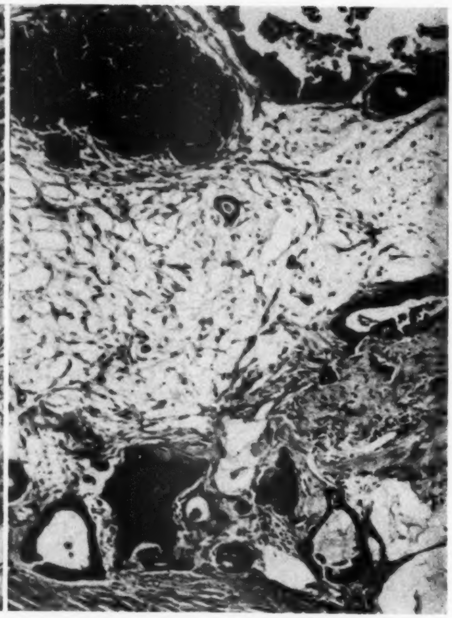

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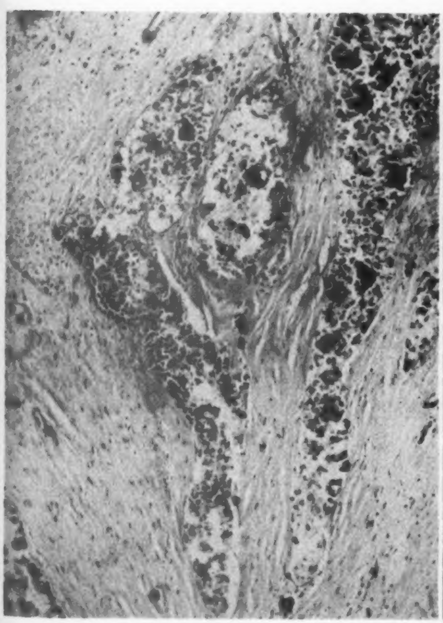

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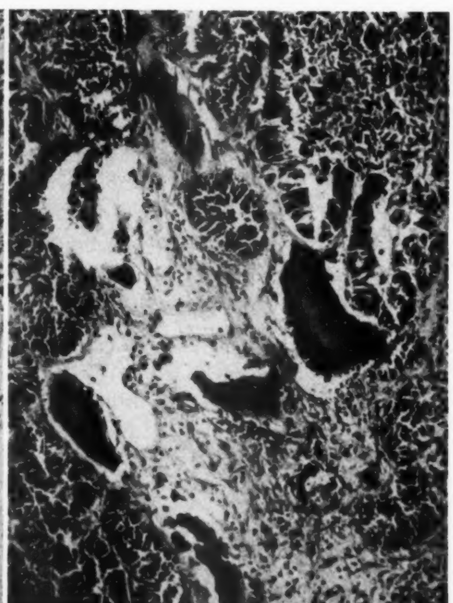

12

ADRENAL NECROSIS AND THROMBOSIS IN ROUTINE NECROPSIES *

Alfred Plaut, M.D.†

From the Laboratory Service of Winter Veterans Administration Hospital, Topeka, Kansas

During a previously reported study of pituitary necrosis in routine necropsies,[1] the thought arose that analogous or similar lesions might occur in the adrenal gland, since the pituitary and adrenal glands share peculiar features that set them apart from other organs.

Material, Methods, and Findings

The adrenal glands of 129 unselected necropsies (adult males) were used. While some were removed in the usual way, in most cases special care was taken to avoid traumatic artefacts. A few transverse incisions were made into the organs before they were fixed in neutralized 10 per cent formalin. After 24 hours' fixation, the organs were freed of fat tissue and sliced. Three or more paraffin blocks were made from each adrenal gland. Sections were cut at 6 or 7 μ and stained with hematoxylin and eosin.

The search for necrosis in the anterior lobe of the pituitary gland can be done under low magnification. It is different with the adrenal cortex. In places where the cells are filled with lipids, one needs high magnification to ascertain the presence of necrosis. Thus, in comparing the occurrence of necrosis in the two organs, one must consider the figures for the adrenal gland as minimum, because small areas of necrosis are easily missed.

The number of sections examined varied from 12 to 200. It was not larger in the positive cases than in the others. Necrosis was found 11 times, 7 times with venous thrombosis, 4 times without it. In 2 additional cases, thrombi were found but no necrosis. In 5 of the 7 cases with necrosis and thrombosis, the amount of necrosis appeared consistent with the extent of thrombosis, but in one adrenal gland with much necrosis thrombi were few, and in another only one thrombus was found in a medium-sized vein. Conversely, one adrenal gland showed large thrombi but no unequivocal necrosis. The possibility of deception by post-mortem changes was carefully excluded. Lesions in

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† Now at: Armed Forces Institute of Pathology, Washington 25, D.C.

necropsies done less than 2 hours after death appeared identical with lesions found after 50 hours.

No necrotic foci were found in the medulla but extensive cortical necrosis occasionally encroached upon it. All cortical layers were affected by necrosis, and its distribution appeared essentially independent of the layers. Small foci were perhaps more frequent in the outer fascicular zone. One large necrotic area occupied the inner fascicular zone, leaving the outer fascicular and the reticular layers intact. Small foci were separated from the capsule by a narrow zone of intact tissue of the glomerular layer; large foci in places reached the capsule. Isolated necrosis was found in protruding cortical nodules.

In the presence of massive thrombosis the necrotic areas reached several centimeters in diameter, sometimes extending through the thickness of the organ. In the absence of thrombosis they generally were less than 2 mm. in size. Their shape was irregular; none were pyramidal or wedge-shaped. The number of necrotic foci could not be determined precisely since this would have entailed cutting and examining an excessive number of sections; sometimes the foci were few, sometimes many. The completeness of necrosis, of which all degrees were encountered, showed no relation to size of focus. The nuclei of blood vessels and of connective tissue often were well stained while the cortical cells showed neither nuclei nor cytoplasmic structures. There was no significant relation between the degree of lipid vacuolation and the occurrence of necrosis.

Attempts at finding a systematic regularity in the occurrence, amount, and distribution of leukocytic infiltration were unsuccessful. Sometimes small portions of a large necrotic area contained leukocytes. This condition deprived me of the principal means by which the age of the necrotic process might have been judged. No heaps or plugs of microorganisms were seen. The aggregations of "lymphocytes and plasma cells," which are so frequent in the adrenal gland, showed no relation to the foci of necrosis. A similar irregularity existed in regard to hyperemia in and around necrotic foci which were not connected with thrombosis: in some very small areas the sinusoids were engorged, while those in the surrounding normal tissue were empty; but another and similar focus had narrow, empty sinusoids. Even in the same section, one focus was found hyperemic, another anemic.

Age distribution was not significant. The main diagnoses were: encephalomalacia (3 cases) and one each of myeloid leukemia; Huntington's chorea with bronchopneumonia; Wernicke's encephalitis

with bronchopneumonia; multiple sclerosis; carcinoma of bronchus; carcinoma of pancreas; myomalacia of heart with pulmonary infarcts; myomalacia of heart with old thrombosis of left renal artery and partial cortical necrosis of right kidney; bilateral hydronephrosis with uremic colitis; rheumatic heart disease with acute necrotizing enterocolitis. The charts gave no evidence of shock in the last weeks of life. The thrombi were located in the larger veins and in muscular portions as well as in portions with a barely perceptible wall. There was no phlebitis and no degenerative change in the wall. No primary capillary thrombi were found. Most thrombi were permeated by endothelial cells, one case showing early, another late fibrosis of a thrombus. In both instances, younger thrombi also were present. The lumina of some veins were bridged by irregular thrombotic masses with broad or filiform attachments to the wall.

Comment

There was no general clinical correlation with the presence of adrenal necrosis, especially no prevalence of hypertension or of endocrine disturbances. The number of cases of encephalomalacia and of degenerative diseases of the central nervous system is not inconsistent with their frequency in the total material. It may be mentioned, without comment, that the two most severe adrenal lesions were found in patients who had severe colitis: the one uremic, the other acute infectious. It is my impression that adrenal necrosis and thrombosis are found more frequently now than in previous decades; but the clinical charts of the patients with necrosis did not show unusual prevalence of modern therapeutic procedures, especially transfusion and use of antibiotics.

The older literature did not discuss the lesions described in this paper. (Observations made in infectious diseases, burns, pregnancy, or the addisonian complex do not belong strictly to this topic and will not be discussed.) The only survey of the subject, to my knowledge, is that made by Mitchell and Angrist[2] in 1943. They found focal necrosis of the adrenal cortex 18 times in 3,080 necropsies. Less than 12 of these lesions, namely, the ones designated as acute, may be analogous to the lesions in my material. Mitchell and Angrist mentioned that "some of the zones of necrosis bore a distinct resemblance to tubercle structures." Such lesions were not included in the present paper. The difference in frequency of necrosis in the two series is striking: 12 in 3,080 against 11 in 129. The high percentage in my material might be explained partly as an accident due to the small size

of the statistical group. Selye[3] stated that "small patches of necrosis and hemorrhage are common in the adrenals of experimental animals and man exposed to various types of acute non-specific damage." Pantothenic acid deficiency in rats (Supplee, Bender, and Kahlenberg[4]) resulted in hemorrhage and necrosis, especially of the reticular zone, often combined with renal necrosis. In acute carbon tetrachloride poisoning, necrosis is often found at the border of the fascicular and reticular zones (Gonzales, Vance, and Helpern[5]).

In the face of such variety of suggested causal factors and lack of morphologic regularity, it appears hopeless to attempt an etiologic interpretation of the described lesions, especially of focal necrosis without thrombosis. A comparison with the cases reported by Mitchell and Angrist[2] will not be helpful because infection or endocrine disorders were present in all but one of their cases, while they play no recognized rôle in my material.

The most obvious explanation for a local necrosis, namely, the shutting off of the arterial blood supply, becomes highly improbable when one considers the arterial system of the organ. The adrenal gland is entered not by three arteries, as most textbooks state, but by as many as 50 or more arterial branches (Anson *et al.*[6]). Adrenal infarction in man on the basis of arterial occlusion has been mentioned in the literature (Furuta[7]) but has not been proved. However, Harrison[8] has obtained areas of necrosis in the fascicular layer of the rabbit's adrenal gland, immediately after ligation of individual arteries.

In one of my cases of bilateral necrosis with thrombosis, the arteries outside the capsule were not remarkable but most arterioles and some small arteries in the cortex showed severe fibrinoid change. The possibility that the narrowing of these vessels was one factor in the causation of the necrosis cannot be excluded, but the intactness of the reticular layer points to other etiologic factors. This patient also had cortical necrosis in the large right kidney while the small left kidney showed much earlier thrombosis of the artery. The coexistence of renal and adrenal necrosis cannot be considered accidental. It has been observed by others in man (McKay *et al.*[9]) and in animals. In another adult male with unilateral renal cortical necrosis, the adrenal glands were normal but there was a large area of necrosis in the anterior pituitary lobe.

None will doubt the causative rôle of large venous thrombi, especially in the adrenal gland with its single vein; but we must not forget that there can be large venous thrombi without necrosis being found even on examination of many blocks. This was true of a 57-year-old

man with carcinoma of a bronchus. The relationship between thrombosis and necrosis is by no means consistent. In a 60-year-old man who died of myeloid leukemia, thrombi were small but necrosis was extensive, and the location of the necrotic areas did not coincide with the leukemic infiltrations. The insufficiency of the purely mechanical explanation is further demonstrated by the observation that the plugging of adrenal veins by tumor is seldom followed by infarction (Oppenheim and Loeper[10]). Hemorrhage and necrosis occur in the adrenal gland together and separately. It is unknown what conditions lead to the one, or the other, or to both. Neither of them shows a significant quantitative relation to thrombosis of veins; and necrosis of arterioles[11] is not a frequent finding.

The idea that adrenal cortical tissue, which is said to have a high oxygen consumption, becomes necrotic because of insufficient oxygenation toward the end of life is theoretically acceptable. However, it does not have the strong appeal which the analogous explanation of incidental pituitary necrosis has, because severe shock is not followed by severe adrenal necrosis, while its causal rôle in severe pituitary necrosis is undisputed.

The fact that adrenal and pituitary necrosis coexisted in 2 cases (78-year-old male with encephalomalacia and rheumatic myocarditis; 67-year-old male with encephalomalacia and obesity) may be accidental. If it is significant, the nature of the connection is not yet understood. Severe post-partum necrosis of the pituitary gland is not accompanied generally by adrenal cortical necrosis. Sheehan and coworkers[12,13] did not mention it. McKay *et al.*[9] have seen it (*loc. cit.*, p. 512). The unknown factors that lead to non-thrombotic necrosis in the adrenal cortex may act directly or by way of the anterior pituitary body as diphtheria toxin does. Adrenal necrosis after injection of diphtheria toxin can be prevented by hypophysectomy (Boguth, Langendorff, and Tonutti[14]). It thus is possible that adrenal necrosis in man might be dependent upon the condition of the pituitary gland.

Further comment can be developed by comparing the two organs. Their similarities are well known (Collin[15]). Both are composed of an endocrine portion and of a modified nervous, neurocrine portion. Both have a complicated blood supply which, in ways we do not understand, is connected with function. Assuming that the blood in the hypophyseal portal vessels flows from the stalk to the pituitary gland, we must conclude that the blood in pituitary capillaries contains less oxygen than that of capillaries whose supply comes entirely from arterioles. Conversely, the blood in adrenal venules and veins may be

better oxygenated than that of other veins since many small arteries traverse the cortex and run directly to the medulla. Many arterial branches, after having passed the capsule, form a capillary network in the cortex. The blood from these capillaries is collected by vessels that open into medullary veins. Thus one may speak of a "portal system" of the adrenal cortex. It differs from other portal systems in the fact that it carries arterial blood; by the same token it somewhat resembles the glomerular system of the kidneys.

One can regard pituitary necrosis found incidentally as a minor degree of the process which, in its full development, leads to the Sheehan syndrome and to Simmond's disease.[1] Adrenal cortical necrosis found incidentally might perhaps, in an analogous way, be related to the severe adrenal lesions as described, e.g., by Arnold, Richer, and Lepore.[16] These cases did not show a significant relation to other diseases, and less than half of them occurred in pregnant women. But, in my opinion, the available data do not yet warrant such an analogy. Judgment in this matter is difficult because the adrenal lesion is not as uniform as pituitary necrosis, but is variously combined with thrombosis and hemorrhage.

The question of how far intravascular fibrin deposition in arterioles and capillaries is responsible for necrosis in the anterior pituitary lobe and the adrenal cortex[9] needs further study. It does not seem to play a rôle in the incidental lesions as reported in this paper for the adrenal gland and in a previous one for the pituitary gland. An obvious analogy is seen in the fact that in both organs necrosis affects only the glandular portion, not the nervous one. And, finally, there is a phylogenetic parallelism. The thick muscle in the adrenal veins and the postnatal change in the inner cortical layers are not found in other mammals. The pituitary gland of man (and of anthropoid apes) also differs from that of other mammals in respect to the reduction of the pars intermedia.[17] Thus both organs obviously are associated with phylogenetic processes.

Summary

In 129 unselected necropsies of adult males, necrosis in the adrenal cortex was found 11 times. In 7 of the 11, adrenal veins contained thrombi. In addition to these 11 cases, there were 2 with thrombosis but without necrosis.

No embolic or thrombotic occlusion of arteries was found.

There was no significant correlation with age, clinical disease, or treatment.

Possible analogies between these adrenal lesions and pituitary necrosis as found in routine necropsies are discussed.

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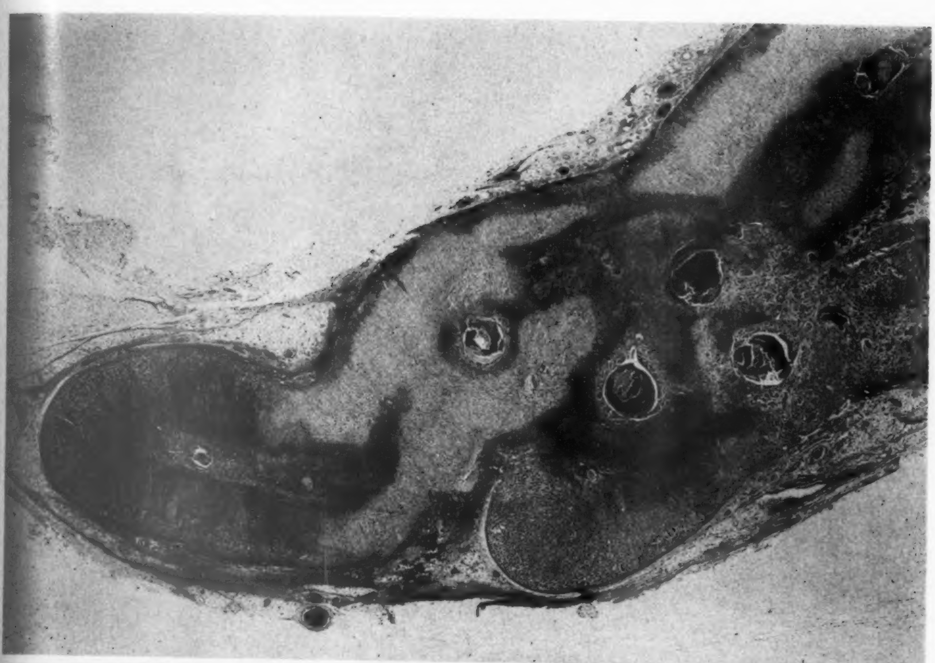
LEGENDS FOR FIGURES

FIG. 1. Large, irregularly shaped areas of necrosis in cortex. The necrosis reaches the capsule in places. The veins are thrombosed. Male, 42 years old, with bilateral hydronephrosis, uremic colitis, and mild diabetes. Hematoxylin and eosin stain. × 8.

FIG. 2. Small area of almost complete necrosis in outer fascicular layer. Surrounding cortical cells contain much lipid. There are no leukocytes. Male, 36 years old, with multiple sclerosis, severe emaciation, and bronchopneumonia. Hematoxylin and van Gieson's stain. × 130.



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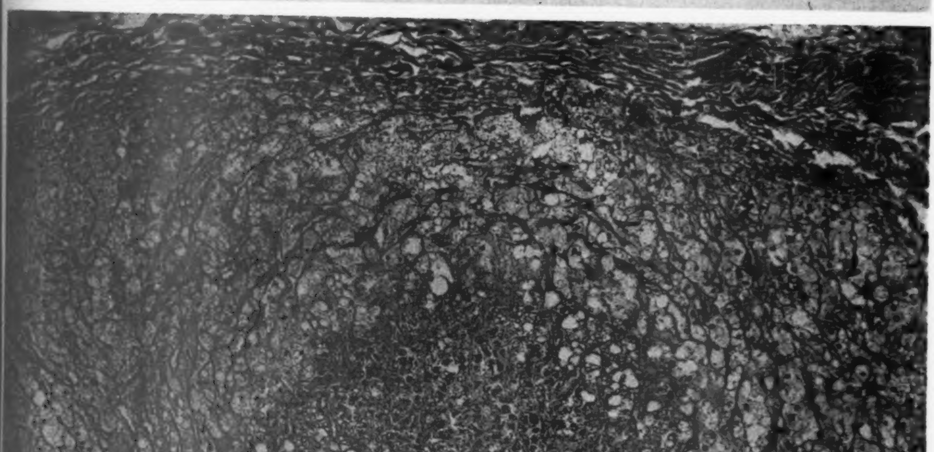

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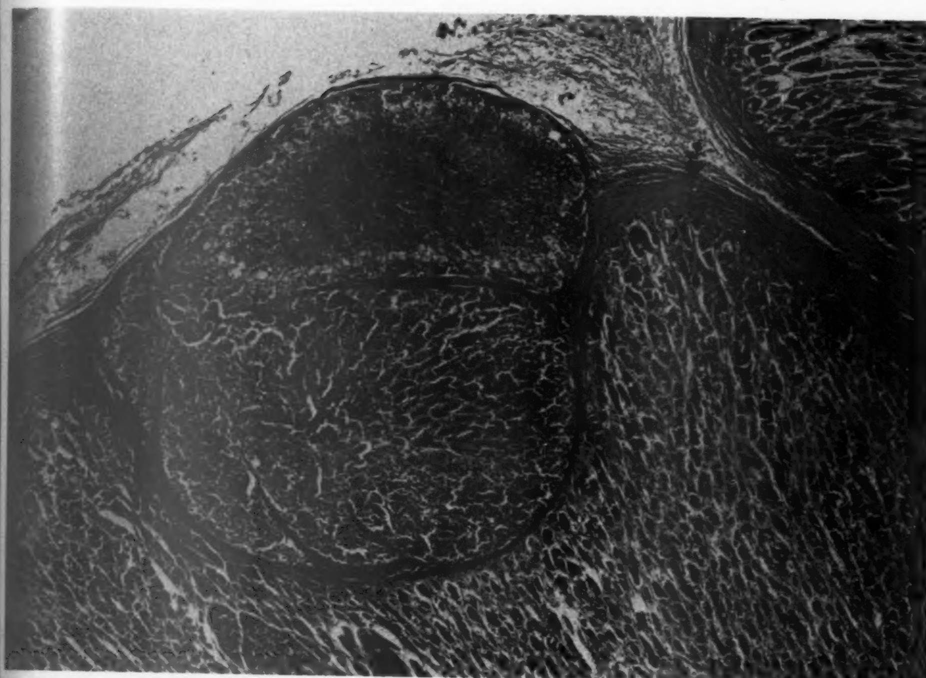
Fig. 3. A protruding nodule, which has separated the layers of the capsule, is necrotic. The surrounding cortical cells contain little lipid. Male, 42 years old, with carcinoma of pancreas. Hematoxylin and eosin stain. × 40.

Fig. 4. Small triangular area of necrosis with fragments of chromatin and with leukocytes. Another necrotic area in the same adrenal gland showed no remnants of nuclei and no leukocytes. Male, 78 years old, with multiple foci of encephalomalacia. Hematoxylin and eosin stain. × 130.



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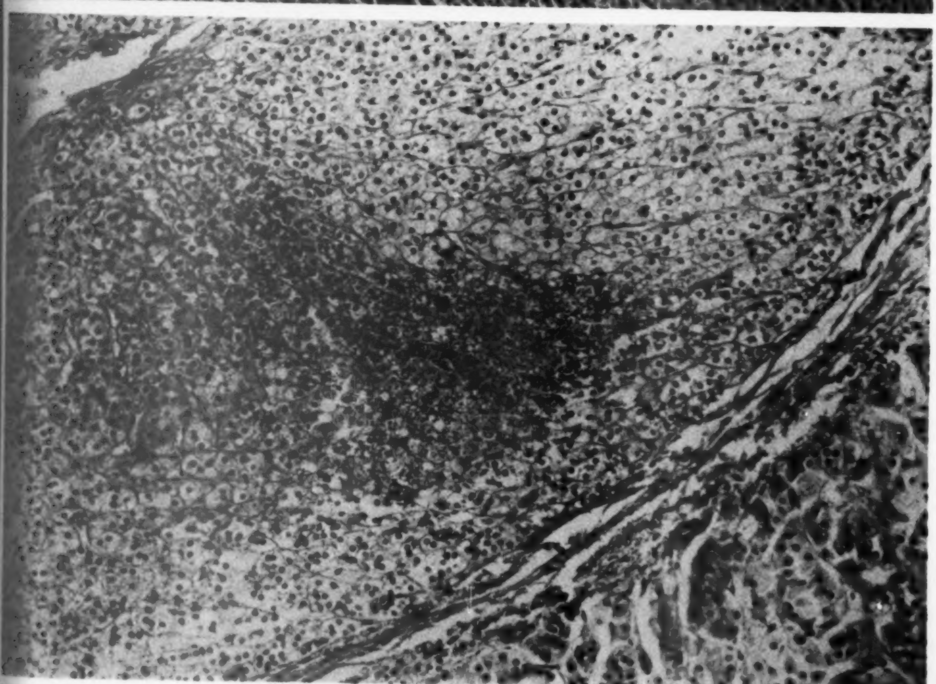

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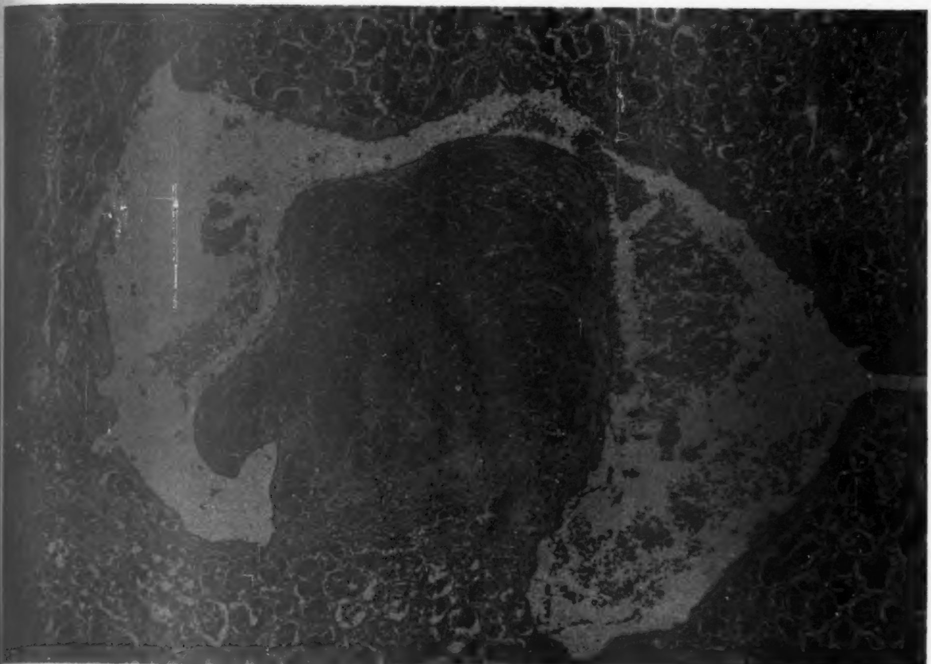
Fig. 5. Irregularly shaped thrombus with beginning organization. It is attached by a broad base to the thin wall of a wide vein. A tributary vein is seen on the right edge of the picture; its mouth is not surrounded by muscle. Male, 69 years old, with encephalomalacia and pneumonia. Hematoxylin and eosin stain. × 75.

Fig. 6. Thrombus, roughly repeating the shape of the lumen of a muscular vein. Thin adhesions connect it with the wall. The mouth of a tributary vein (left lower quadrant) is surrounded by muscle. Male, 54 years old, with bronchiectasis and Wernicke's polio-encephalitis. Hematoxylin and eosin stain. × 120. See also: Figure 6, *Am. J. Path.*, 1952, **28,** 899.



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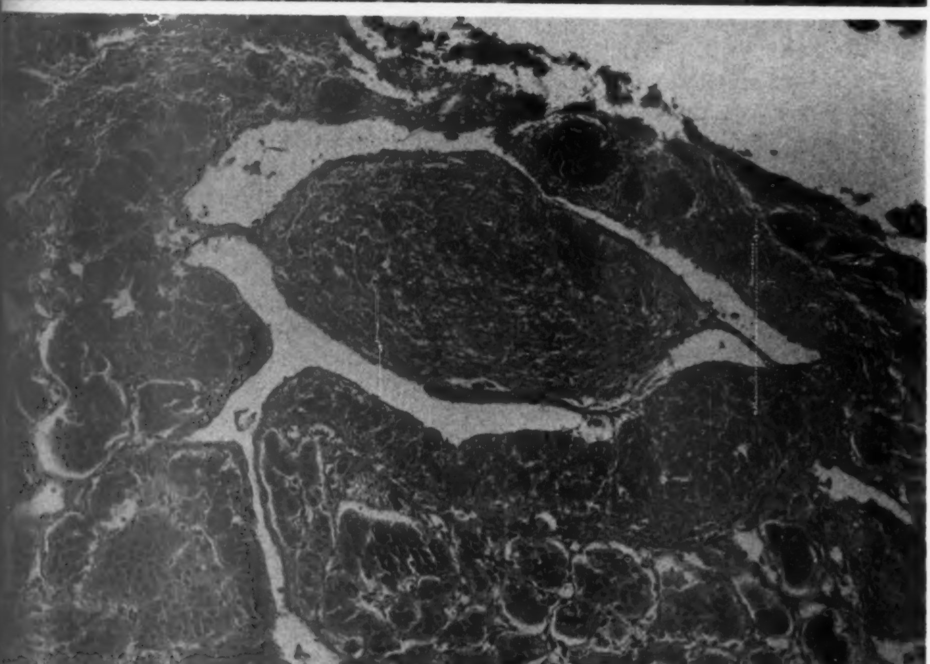

6

INTIMAL REPAIR OF THE AORTA OF THE RABBIT FOLLOWING EXPERIMENTAL TRAUMA *

John T. Prior, M.D., and Robert V. P. Hutter, M.D.†

From the Department of Pathology, State University of New York, Upstate Medical Center, Syracuse, N.Y.

Despite the fact that arteriosclerosis is the leading cause of morbidity and mortality today, one cannot avoid the conclusion that our knowledge of its pathogenesis is shamefully deficient. As we have suggested previously,[1] this is in a large measure due to overemphasis of the rôle and mechanism of lipid deposition, directly attributable to the production of experimental "atherosclerosis" by the administration of cholesterol to a variety of animals. With the main interest focused upon the method of lipid transport through the vascular endothelium, it is understandable why the basic processes of injury and repair within the arterial subendothelial zone have been ignored.

The present investigation was stimulated by our observation of microscopic thrombi associated with intimal thickening in the aortas of 50 consecutive necropsies from the pediatric service.[1] Originally, these thrombi were an accidental microscopic finding and were noted primarily in those cases in which there had been a bacteremia (Fig. 1). Although Rokitansky[2] had originally pointed out that endothelial thrombi might be the basis of plaque formation in atherosclerosis, the idea was soon discarded and only recently has there been a reversion to this early concept. Duguid[3-6] and Crawford and Levene[7] have shown that mural thrombosis is extremely common in the aorta and that appearances identical with those seen in atherosclerosis may result from organization of such thrombi. In view of these observations it seemed pertinent to study the reaction to artificially-induced aortic intimal trauma in the experimental animal and to trace the sequence of repair eventuating in complete healing.

Methods

Thirty New Zealand white rabbits, approximately evenly divided as to sex, were used in this study. Their average age at the beginning of the experiment was about 10 weeks and average weight was about 3500 gm. They were housed separately in wire cages and were fed Purina rabbit chow and water ad libitum.

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† Now at: Department of Pathology, Yale University School of Medicine, New Haven, Conn.

The animals were anesthetized with pentothal and ether and the peritoneal cavity was incised under sterile precautions. The abdominal viscera were retracted from a segment of lumbar aorta and the latter was freed from the adjacent connective tissue with a minimum of trauma. The aorta was then pierced at an oblique angle with a 24-gauge needle, the point of which was deliberately angulated to provide a barbed appearance. The intimal surface was then traumatized by repeated vertical and horizontal motions. The needle was withdrawn and hemostasis effected by means of direct pressure over the puncture site. Before closing the peritoneal cavity the area of trauma was marked by silk sutures in the periaortic connective tissue.

The animals were sacrificed at approximately weekly intervals ranging from 3 hours to 220 days following intimal injury. Within the experimental series, the entire aorta was removed and areas at a distance from the traumatized segment were considered normal and to serve as controls.

Pathologic Findings

The technique followed in this experiment did not permit control of the depth of the trauma, so that in any vessel the injured tissues might vary from the intimal coat only to the entire vessel thickness including the adventitia. In general, we were more interested in areas in which the trauma had been superficial, i.e., intimal only.

Normal rabbits of this age possess an unusually thin intimal coat, the endothelial lining appearing to lie directly upon a well developed internal elastic membrane. Isolated fibroblasts can, however, be recognized within the subendothelial zone. Animals sacrificed 3 days following intimal injury disclosed pitted lesions grossly and showed no evidence of intimal thickening. Microscopically, in those areas in which the trauma was relatively superficial, there was deposition of loosely arranged fibrin in which nuclear débris and rare mononuclear cells could be recognized (Fig. 2). The endothelial cells adjacent to the injured area were swollen and hyperchromatic and, where the injured segment was narrow, beginning endothelization was apparent. Below the fibrin layer fibroblasts were arranged in a parallel fashion, generally perpendicular to the endothelium. Polymorphonuclear leukocytes (amphophils) were abundant within this zone. The deeper, intact media showed edema and the adventitia invariably disclosed early fat necrosis, presumably the result of the pressure applied for hemostasis.

At 7 days the intimal surface was characterized by multiple, shallow, eroded areas giving the vessel a pebbly appearance. Microscopi-

cally, endothelial activity was much more prominent and it was not possible to distinguish these cells morphologically from the active fibroblasts growing toward the surface from the inner media (Figs. 3 and 4). Cells of both types were arranged in a disorderly fashion and were characterized by cytoplasmic swelling and nuclear hyperchromatism without evidence of mitosis. Beneath this zone in some areas the young fibroblasts had completely replaced the fibrin while in other areas the fibrin, although covered, had a peculiar pale pink "fibrinoid" appearance (Fig. 3). Calcification of these fibrinoid zones sometimes followed, being observed as early as 49 days in one case (Fig. 6). About the margins of the fibrinoid areas a mononuclear cellular reaction was noted frequently. Intramural hemorrhage was observed at this stage but new capillaries were not in evidence. In the adventitial foci of fat necrosis a giant cell reaction and large numbers of lymphocytes and plasma cells were present. Occasionally, the non-organized fibrin protruded into the vessel lumen as an endothelium-covered polypoid excrescence (Fig. 5). Although there was evidence of fibroblastic proliferation at the base of such nodules, calcification occasionally supervened before organization was completed (Fig. 7).

At 18 days following intimal trauma, the same pitted lesions as have been described were seen on the intimal surface, but beginning thickening of the adjacent margins was present. Microscopically, the lesions showed no significant changes from those described at 7 days. The fibroblastic reparative process was nearly completely avascular and no unusual changes within the vasa vasorum were apparent.

At 35 days following injury, the aortic intima showed raised, somewhat yellow foci above and below the lacerated segment. These superficially resembled the plaques of adult atherosclerosis. Microscopic examination of these thickenings revealed them to be conchoidal and composed of loose connective tissue (Fig. 8). The cells comprising the thickened focus were slender, spindle-shaped, and hyperchromatic, and showed moderate variation in polarity. The internal elastic membrane at the base of the thickening frequently showed fraying, fragmentation, and splitting. The portion of the intima which was contiguous with the internal elastic membrane disclosed considerable edema and nuclear débris. With Verhoeff's technique these intimal plaques were found to be composed predominantly of elastic tissue (Figs. 9 and 10), which appeared as delicate fibrils arranged parallel to the internal elastic membrane and which seemed to be most dense in the more superficial portion of the intimal thickening. Masson's trichrome stain revealed only minute amounts of collagenous material.

It is this stage of the reparative process which is morphologically inseparable from the fibro-elastic intimal thickenings described by Prior and Jones[1] and which occurred so consistently in children over the age of 2 weeks that we postulated that these might actually represent the earliest phase in the development of the atheromatous plaque in man. An impressive gross lesion on the intimal surface of the rabbit's aorta was not noted until 61 days following injury. At the site of trauma numerous transverse lacerations, each measuring 0.1 cm. long, were observed. The intimal surface about the margins of these lacerations presented a raised, slightly yellow, rolled appearance. The entire area was placed in a solution of 50 per cent alcohol to which several drops of Herxheimer's scarlet red had been added, but these yellow areas failed to stain positively for lipid material. The microscopic picture of these rolled, raised areas was similar to that of the thickenings noted at 35 days (Fig. 7), although the trauma had been deeper so that the internal elastic membrane was partially replaced by dense fibrous tissue. Wherever the medial elastic lamellae were injured they were replaced by non-specific dense scar tissue with a reaction of mononuclear type.

At longer intervals the gross appearance of the aortic intima was striking, the typical surface alterations being shown in Figure 11. Large plaque-like elevations were located about the margins of previously injured segments. Although these areas had a yellowish discoloration, they also failed to stain positively with the alcoholic Herxheimer's scarlet red solution. The plaques averaged about 0.1 cm. in diameter with the largest measuring 0.3 cm., and their elongation was invariably in the longitudinal plane. Microscopically, the thickenings were composed of mature connective tissue which was rich in elastic tissue. Such an area of thickening 148 days following injury is shown in Figure 12. The reduplication or splitting of the internal elastic membrane was striking and minute amounts of lipid material were visible as demonstrated by osmic acid preparations. The fat was sparse and located within the more superficial portion of the thickening. No foam cells were recognized and this fatty material appeared to be entirely extracellular. Of considerable interest is the fact that only the transverse lacerations remained as depressions and became associated with these fibro-elastic thickenings and that no residual scarring from the vertical lacerations could be identified. Although reasons for this are not clear, one is tempted to suggest that the transverse lacerations may have been associated with disturbances in the flow, such as eddy currents.

Discussion

The pathogenesis of atherosclerotic plaques is a neglected subject in the standard textbooks of pathology. The historical background of their development is a succession of theories ranging from a simple chronic inflammatory concept to one in which the physicochemical changes within the intimal extracellular colloids are paramount, and, finally, to the present concept centered upon aberrations of serum lipids and lipoproteins. It is of interest that Duguid[3] has pointed out that Rokitansky[2] first held that the atheroma was produced by the deposition of an endogenous product derived from the blood and, for the most part, from the fibrin of the blood. He noted the metamorphosis of the deposit into a pulpy mass consisting of crystals of cholesterin, fatty globules, and of molecules of albumin and calcareous salts. Virchow[8] later refuted this concept, holding that the connective tissue proliferation was initially subendothelial, and referred to the endothelium as if it were a fixed landmark by which the tissues of the vessel could be identified.

Duguid's views[3-6] are in essence a reversion to the teachings of Rokitansky[2] and appear to contrast sharply with the present day concept of the genesis of the plaque. Karsner,[9] for example, in discussing the pathogenesis of atherosclerosis, stated that the first lesion is damage in the lower intima with splitting of the elastic fibers, some destruction of fibrous connective tissue, and lipid deposition. In response to the injury, connective tissue is formed in excess to produce intimal plaques. This overgrowth of fibrous tissue is succeeded by hyalinization, and either before, or coincident with hyalinization, there may be mucoid degeneration of connective tissue. Duguid[3] pointed out that when a thrombus forms in an artery it adheres to the wall, the endothelium disappears, and there is an invasion of the mass by connective tissue. There then follows a progressive transformation of the outer layers of the thrombus into fibrous tissue, so that an advancing zone of fibrosis is formed which overruns and obliterates the original line of demarcation between thrombus and intima—an appearance similar to that seen in pleural or pericardial inflammation. Organization of the thrombus then follows, although the progress of this change depends to a great extent upon the composition of the mass. Pure fibrin, which is relatively firm, is readily organized, but collections of corpuscles, such as are present in red thrombi, undergo softening and fatty degeneration which interfere with organization. The corpuscles break down and globules of fat appear among them

and accumulate until they obscure the entire semi-fluid or paste-like mass. Such masses, having no firm structure on which granulation tissue can build, tend to persist as areas of fatty degeneration and it appears that this is one of the ways in which atheromatous plaques are formed. Fatty changes also occur in the fibrinous parts of the thrombus, but in these areas the thrombus usually is not associated with softening and therefore is not so permanent. With transformation of fibrin into fibrous tissue the fats are taken up by phagocytes which tend to occur in clusters around the zone of organization, and with canalization and further organization they appear to be embedded in a thickened intima. Duguid[6] contended that these observations can be confirmed by inducing experimental thrombosis in animals by such methods as pricking the central artery of the rabbit's ear or by passing silk threads a short distance along the lumina of the femoral or carotid arteries of the dog. He also called attention to Harrison's[10] study in which fibrin particles were injected into the veins of rabbits. The particles which lodged in the smaller pulmonary arteries were converted into fibrous thickenings indistinguishable morphologically from the vascular changes in human pulmonary hypertension. Finally, it would appear that the study of aortic fibrin deposits by Crawford and Levene[7] adds strong support to Duguid's hypothesis.

Our main interest has been with the non-lipid-induced variants of arteriosclerosis. Reviewing the literature, one finds a kaleidoscope of experimental methods of inducing vascular lesions, both similar and unlike those found in human blood vessels. Broadly, these may be classified under chemical methods (including macromolecular substances), and physical (including direct and indirect trauma). Anitschkow,[11] in his survey of the literature (1890–1924), cited many examples of mechanically induced arterial lesions. All of these procedures—ligating, pulling, pinching, wounding, cauterizing with galvanic current or silver nitrate—produced inflammatory responses which were not similar to human arteriosclerosis. These procedures did, however, result in increased permeability of the vascular intima to intravenous trypan blue dye, and subsequent intimal thickening. Ssolowjew[12] showed that the course of the arterial lesion depends upon the degree of injury to the elastic framework. With severe injury the adventitia initiates granulation tissue replacement of the structure. With the elastic framework intact (i.e., only the cellular parts destroyed) the regenerative proliferation begins in the adjacent intact media. Contributions by the intima and intra-luminal circulating cells are also said to have a rôle. Ssolowjew demonstrated that

isolated transverse lacerations of the elastic lamellae of the inner media, produced by fixation of the carotid artery into a bridge of skin, rendered the overlying intima more permeable to substances circulating in the blood (trypan blue and lipid substances in cholesterol hyperlipemia). The reparative processes consisted of subendothelial thickening by cells migrating from the intima. Altschul[13] spoke of migration of smooth muscle cells across the elastic lamina to the intima. He also referred to Krafka,[14] who contended that there is a migration of intimal cells to the media through herniations in the internal elastic lamellae, and that in the later stages of repair associated with new elastic and collagen fibers, lipids are no longer deposited at sites of intimal tearing.

Dill and associates[15,16] studied aortas of rabbits with constricting rings located both proximally, and proximally and distally, to the renal arteries. The added factor of pregnancy was present in some of these animals. They noted in the thoracic and abdominal aorta atheromatous plaques which were composed of amorphous intimal material which stained positively for fat, and fibrillation of the underlying elastic lamella. They concluded that the incidence of plaques was closely related to the level of systemic blood pressure and to the length of time such pressure acts. No lesions were seen below the level of the constricting band nor were any present in those animals with the band but without elevated blood pressure. Plaque formation was observed much more frequently in animals which were pregnant. They concluded that an elevation in aortic pressure transmitted through the depth of the wall would interfere with filling of the vasa vasorum and bring about an anemia of the subintimal layers—a hypothesis based upon the observations of Winternitz, Thomas, and LeCompte.[17] Mehrotra,[18] who recently investigated changes that occur in ligated arteries and veins, noted that double ligation of arteries results in obliteration of the lumen by intimal proliferation, with subsequent medial atrophy. Double ligation of veins also stimulates intimal proliferation; however, a new lumen with connection to other veins and the re-establishment of circulation always occurs. It was believed that the relative ease of venous repair contrasted with arterial repair could be explained by structural differences in the vessels, veins being composed chiefly of fibrous tissue while arteries are rich in the more highly differentiated muscle and elastic tissues.

Searing the adventitia of the ascending aorta of dogs produced changes in the outer and middle portions of media.[19] These consisted of necrosis, liquefaction, cyst formation, and ultimate replacement by

collagenous tissue. The rate of necrosis was determined by the extent of the collateral circulation. In one animal there was spontaneous rupture and subsequent dissection of an aneurysm. In an attempt to induce atheromas or dissecting aneurysms, blood was injected into the vessel walls of dogs with the result that after 3 months there was either complete healing or medial scarring without evidence of hemorrhage or dissection.[20] Closely allied to this discussion of injury and repair of arterial walls is the progression of events seen in arterial homografts. Swan, Robertson, and Johnson,[21] in a study of aortic transplants in dogs, observed that the adventitial and intimal tissues were totally replaced by the host. In grafts stored less than 40 days, up to 50 per cent of the muscle of the media survived, while none survived in grafts stored over 40 days although the elastic tissue survived. They also noted that after 5 months the intima showed spotty areas of calcification and some lipid deposition, presumably cholesterol.

Finally, a pertinent study is that of Sheehan[22] upon the effects of irradiation on small arteries (100 to 500 μ in diameter). He described foam cell plaques in the intima of irradiated human uterine, ovarian, and rectal arteries. The plaques were located between the endothelium and the internal elastic lamina and were composed of either foam cells alone or foam cells in combination with other cells, fluid, fibrin, and hyaline material. Pathologic changes in the media and adventitia were not constant. The plaques produced marked narrowing or occlusion of the vessel lumen, "thrombosis, fibroblastic proliferation of the intima or deposition of elastic tissue in the thickened intima seldom result." Nevertheless, Sheehan illustrated segments of a uterine artery with necrosis adjacent to the radionecrotic area at one end, a plaque at the other, and a thrombus in the intervening portion. He proposed the theory that radiation altered the permeability of the endothelium, facilitating migration of lymphocytes, monocytes, and erythrocytes into the intima. With red cell degeneration, liberation of their lipids and subsequent ingestion of lipids, foam cells are produced. No stains for fat were done, but since crystals, presumably cholesterol, accompanied the foam cells, it was inferred that the foam cells contained cholesterol and were really xanthoma cells.

Summary

The reaction to a form of mechanically induced trauma to the intimal surface of the rabbit aorta has been described. The injured segment heals chiefly by means of marked fibroblastic activity, resulting in the production of a localized fibro-elastic thickening within which,

in the later stages, minute amounts of lipid were demonstrated. Although the gross and microscopic appearances of these intimal "plaques" showed little similarity to human atherosclerotic plaques, the lesions were histologically inseparable from the fibro-elastic aortic thickenings observed in infants, which are considered by some to be precursors of the adult atheromatous lesion. A brief review of the results after other forms of mechanically induced vascular trauma has been presented.

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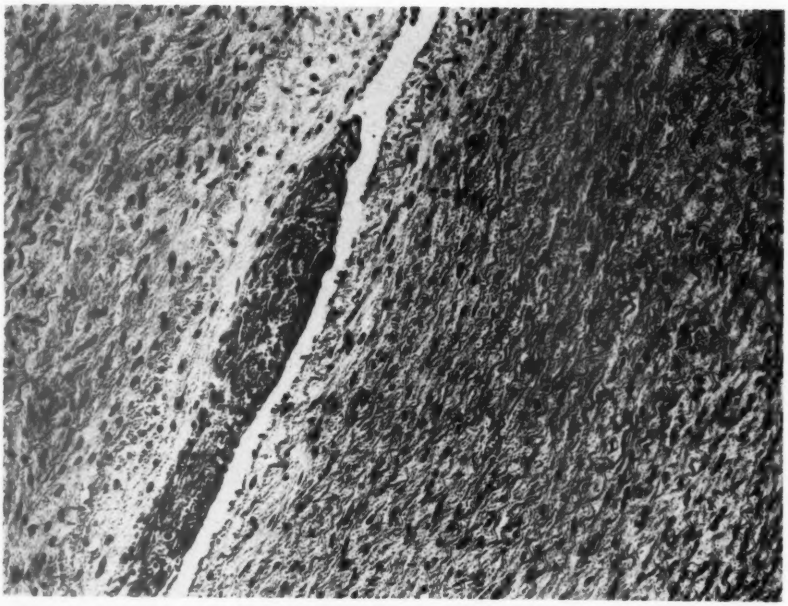
LEGENDS FOR FIGURES

Fig. 1. Aortic lumen of an 8-year-old child showing, on the right, intima of normal thickness, and, opposite this, a fibro-elastic plaque with superimposed thrombus. × 175.

Fig. 2. Traumatized segment of a rabbit's aorta after 3 days. A meshwork of fibrin and proliferating fibroblasts is growing toward the lumen. × 465.



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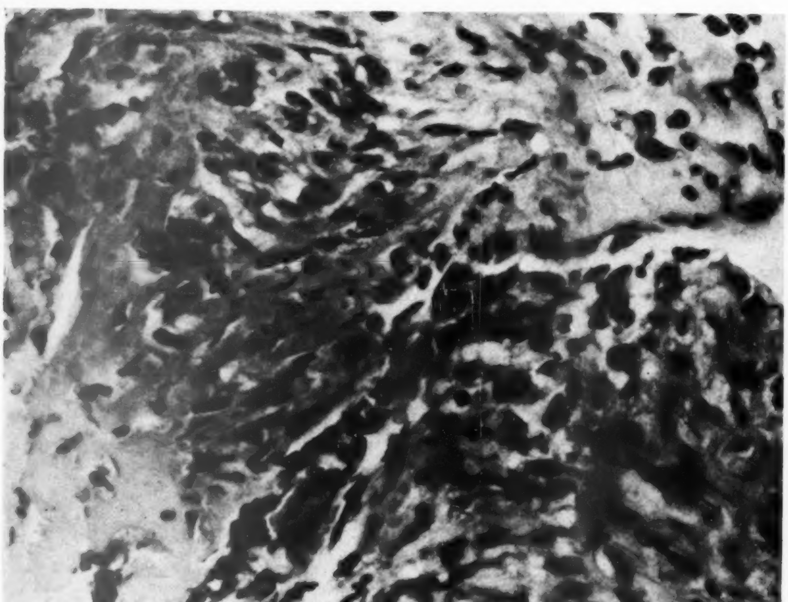

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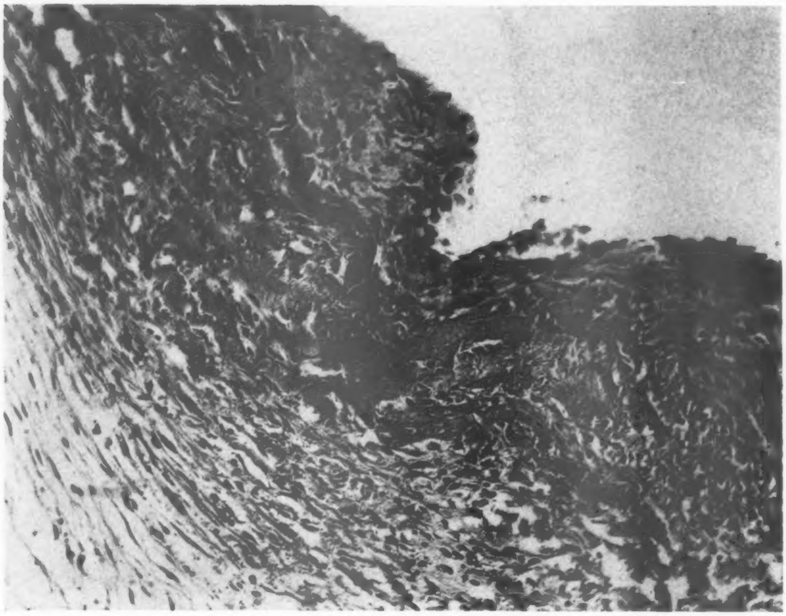

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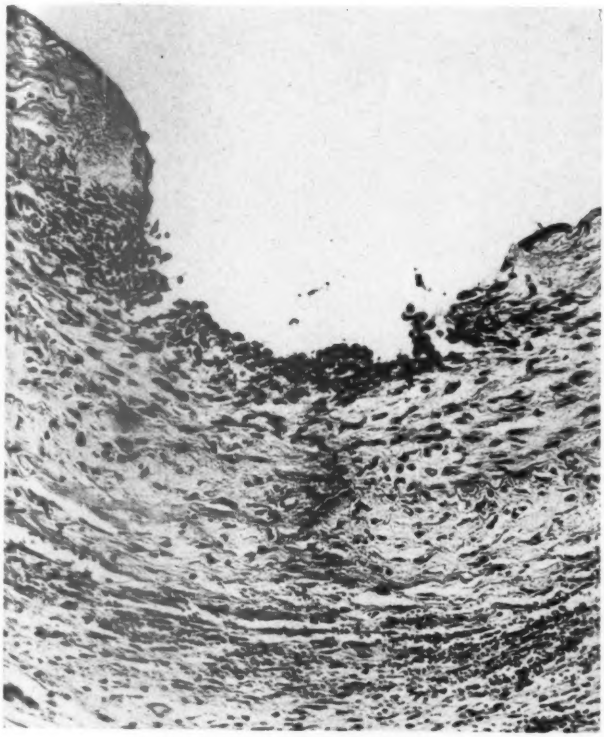
FIG. 3. Traumatized segment at 7 days, showing fibroblastic hyperplasia on either side of the central depression. Fibrinoid material is present within the media. × 160.

FIG. 4. Traumatized segment at 7 days, showing extensive fibroblastic and endothelial cell proliferation. × 160.

FIG. 5. Traumatized segment at 7 days, revealing the polypoid non-organized mass at one margin of the depressed area. × 160.



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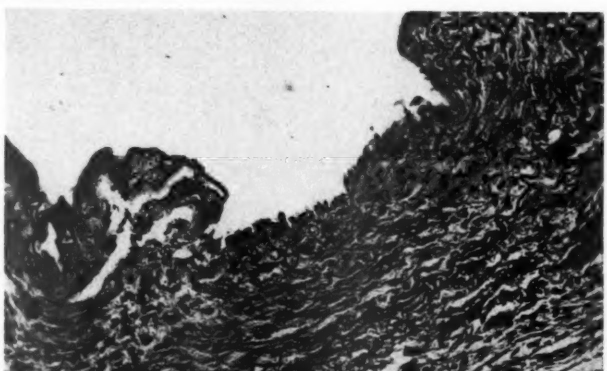

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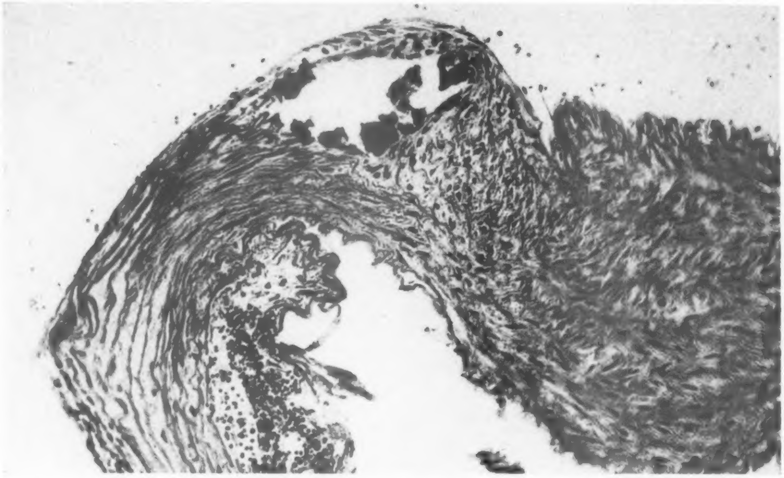

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FIG. 6. Traumatized segment at 49 days, with calcification of the non-organized material. × 160.

FIG. 7. Calcification of a polypoid mass similar to that shown in Figure 6, at 111 days. × 160.

FIG. 8. Thickened fibrous intimal plaque 35 days after injury. The internal elastic membrane shows fraying toward the right. × 465.

FIG. 9. Elastic tissue stain of a plaque similar to that shown in Figure 8. × 165.

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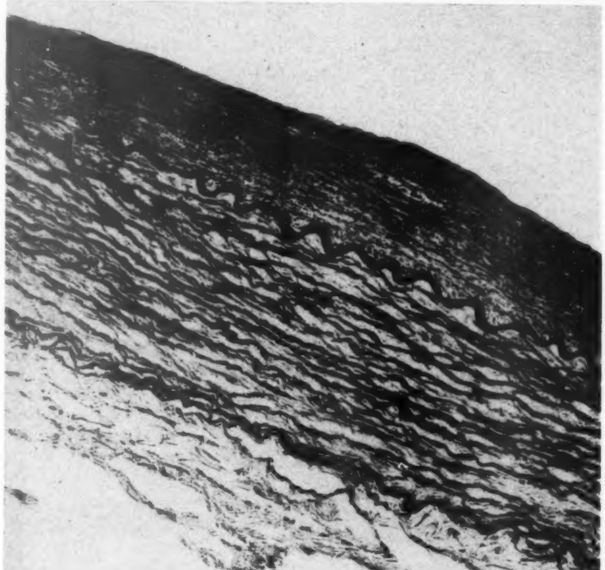

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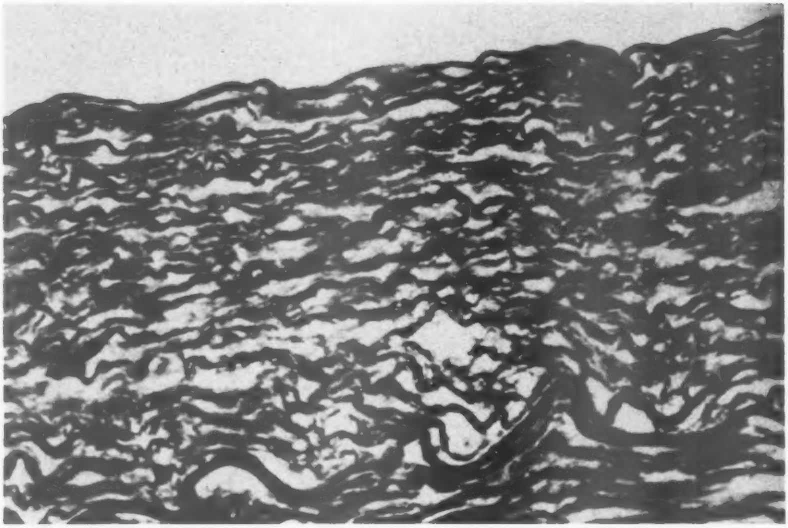

FIG. 10. High-power magnification of the plaque shown in Figure 9. × 600.

FIG. 11. Intimal surface of the aorta 163 days after injury, showing the intimal thickenings adjacent to the transverse fissures. × 5.

FIG. 12. Preparation stained with osmic acid to show the distribution of lipids in the subendothelial zone 148 days after injury.

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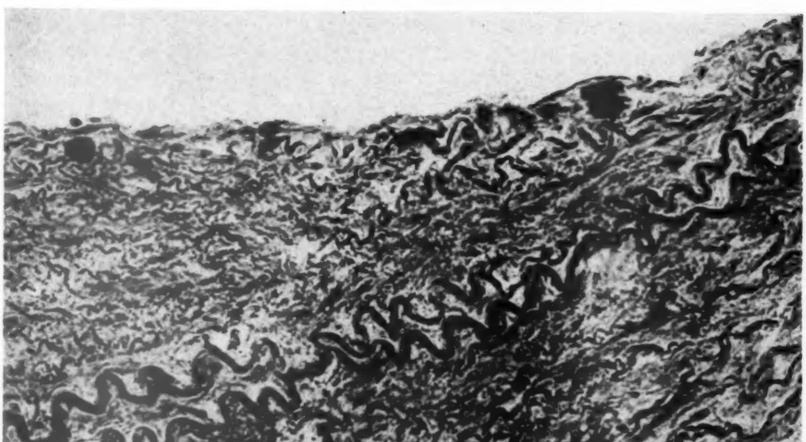

12

TISSUE CULTURE OF HUMAN BREAST CARCINOMA *

MARY FAITH ORR, M.A., and BARTON McSWAIN, M.D.
From the Department of Surgery, Vanderbilt University School of Medicine, Nashville, Tenn.

Carcinomas of the mammary gland have been the subject of numerous and extensive fundamental studies in tissue culture. Tumors of laboratory animals such as the mouse have been used primarily. Lewis and Strong,[1] Santesson,[2] and others have conducted such studies. The work on tissue cultures of the human breast is limited to a few investigations. Cameron and Chambers[3] successfully cultivated epithelium from "infiltrating duct-cell" carcinomas but had no success with tumors that were extensively fibrous. Coman[4] and Royle[5] reported outgrowths of fibroblasts and macrophages, and irregular cords and clusters of epithelium from carcinomas (not classified) of the breast. Many workers are of the opinion that it is difficult to obtain adequate results from human mammary cancer. It becomes obvious, therefore, that a systematic and thorough study of the cultural characteristics of various human breast carcinomas is needed.

The present investigation was undertaken in the hope of beginning such a study. Tissue cultures were made of a series of primary carcinomas of the human female breast, using cultural conditions that will be described. The results of these tissue cultures will be given in this report.

MATERIALS AND METHODS

Immediately after removal, specimens of primary carcinomas of the breast were cut into fragments about 1 to 2 mm. square, care being exercised to cut away fat and large pieces of connective tissue. The fragments were washed in rapid changes of balanced salt solution until the solution was clear. Normal breast tissue was treated in the same manner.

The media for this study were fresh heparinized chicken plasma, 10-day-old chick embryo extract, and a nutrient solution composed of 50 per cent Hanks's salt solution, 47 per cent pleural or ascitic fluid, 3 per cent embryo extract, and 500 units of penicillin G per cc. All media had a pH of 7.4 to 7.6.

Carrel flask (D 3.5) and roller tube methods were chosen for this investigation. Preparation of the Carrel flask consisted of planting six fragments of tissue in a clot composed of equal parts of chicken

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plasma and 10-day-old chick embryo extract. After allowing the clot to become firm, 1 ml. of nutrient solution was introduced and the flask closed with a rubber stopper. Roller tubes were made by clotting three or four fragments on a no. 1, 12 by 50 mm. coverglass. This preparation was placed in a 16 by 150 mm. pyrex test tube. Two ml. of nutrient solution were added. One half of the tubes, closed with rubber stoppers, were put into a roller drum apparatus rotating ten times an hour. One half of them were slanted in a fixed position so that they would be comparable with the Carrel flasks. Cultures were incubated at 36° C.

After the first signs of outgrowth were observed, the nutrient solution was renewed routinely once a week. Where liquefaction of the clot occurred, a patch was made by removing the fluid and applying new plasma and embryo extract. Subculturing depended upon the type of outgrowth. Fibroblasts were subcultured as needed for the health of the cells. Epithelial outgrowths were very seldom disturbed. Carrel flasks were used for maintaining cultures for long periods, while roller tube cultures were primarily used for staining at various intervals. Daily observations were made of all living cultures.

A staining method devised by W. Jacobson, employing the Giemsa and May-Greenwald stains, was used for slide cultures.[6] Histologic sections were prepared in the usual manner and stained with hematoxylin and eosin.

Results and Observations

When sterile specimens of cellular, non-necrotic portions of tumors were available, good results were obtained with the cultural conditions described. Poor results were caused by contamination, trauma, or removal of an unsatisfactory portion of tumor. Outgrowths were evident between the fourth and sixth days of cultivation. Both Carrel flasks and roller tubes produced similar results.

Fibroblasts in the presence and in the absence of epithelium presented the typical picture of a connective tissue culture. They were mostly (Fig. 1) masses of spindle-shaped cells radiating from the explant and often forming reticular patterns. The nuclei varied in size, were oval, and usually located in the broad part of the cell. Nucleolar material was variable in amount and shape (Fig. 2). The cytoplasm had a homogeneous appearance except for occasional small granules and vacuoles. Mitotic figures were frequent. Propagation of these cells was relatively easy since they developed into dense outgrowths, did not liquefy the clot, and were stimulated by tissue culture media and techniques. The presence or absence of fibroblasts did not seem

to affect the epithelial formations in primary cultures, although they sometimes created barriers. They proliferated more rapidly and existed much longer than the epithelial cells.

Epithelium migrates in a variety of forms under conditions of life *in vitro*. It forms sheets, broken sheets, cords or tongues, and isolated groups of cells. Undifferentiated sheets of cells, relatively free of fibroblasts, often migrated from the explants (Fig. 3). These sheets tended to liquefy the clot and became attached to the coverglass. Three or 4 days after the appearance of the epithelial cells, migration stopped, but the outgrowth could be maintained for 1 to 4 months in good condition by changing the fluid nutrient regularly. Some of these sheets rolled at the edges and finally withdrew into a solid mass. Addition of another clot did not prevent this occurrence, as the clots were repeatedly liquefied. In some cases the patches were never successful once the epithelial cells had reached their limit and relatively few fibroblasts were present. Other sheets tended to separate into groups of cells (Fig. 4); eventually these would either float away or disintegrate. These sheets of epithelial cells were never more than one cell in thickness. A few successful subcultures were made, but these attempts usually resulted in either a rolling up of the cells with no growth or complete destruction in trying to loosen the cells from the coverglass. These efforts were discontinued so that the cultures could be maintained for study.

Cords or tongues of epithelium also appeared in the outgrowths (Figs. 5 and 6). These formed both in sparse and in dense outgrowths of fibroblasts. They appeared to be more solid than the sheets, which were very loose and separated easily. Furthermore, less liquefaction was observed.

Groups of epithelial cells sometimes developed some distance from the explant. Figures 7 and 8 are representative of such groups of cells which also contained multinucleated cells. These groups were not the result of a separating sheet of cells. They were surrounded by dense growths of fibroblasts, although the area and adjacent zone may be free of them. It seemed as though the proteolytic activity of the cells created a zone through which the fibroblasts could not grow.

In many cases, fibroblasts and epithelial cells migrated simultaneously with a dense outgrowth of fibroblasts on one side and a sheet of cells on the other. The fibroblasts could be subcultured and kept proliferating, but the epithelium usually remained on the coverglass. The most successful technique at present to separate the epithelium and fibroblasts is to peel away the clot containing the explant and fibro-

blasts, leaving the epithelium behind. Again, the epithelium could be maintained but did not show an increase in cell numbers.

Some cultures produced distinctively different outgrowths where fibroblasts and epithelial cells emerged in the same zone. The epithelium was a clearly recognizable sheet while well populated with fibroblasts. These outgrowths of epithelium were more compact than outgrowths having no fibroblasts. Less rapid liquefaction was seen in these cultures, probably because some of the fibroblasts were growing into the clot above the epithelium which was near the glass.

Certain cytologic observations were made. For comparison, three specimens of normal breast tissue were cultivated under the same conditions as tumorous tissue. Two of these produced epithelial cells in the outgrowth. The epithelial sheet (Fig. 9) was made up of cells with lightly stained, inconspicuous cytoplasm. The nuclei varied somewhat in size but had smooth, regular membranes and contained one or more small nucleoli. Although the cytologic features can vary, epithelium from the tumors (Fig. 10) stained much more densely than that of the normal tissue. The neoplastic cells varied in size, assumed varied shapes, and tended to separate. The nuclei were of different sizes and frequently had irregular membranes. The nucleolar material increased but was variable in amount and shape. No fragmentation of the nuclei was observed but multinucleated cells were seen occasionally.

Only a few of the tumors produced epithelial outgrowths that contained frequent mitotic figures. Many of these figures had a very abnormal appearance (Fig. 11).

As a matter of further interest, preliminary comparisons were made between *in vivo* and *in vitro* similarities of epithelial formations and their cytologic aspects. For this purpose, two adenocarcinomas (Figs. 12, 13, 14, 17, and 18) and their tissue cultures (Figs. 15, 16, and 5) were used. A comparison of the observations is presented and the significance discussed in the following section.

Discussion

An unselected series of primary carcinomas (Table I) of the human female breast were cultivated so that the procedure and composition of media would not be factors influencing the behavior of the outgrowths. Figures 19, 20, 21, and 22 are representative of the different types of tissue cultured. Specimens were composed chiefly of epithelial cells and stroma; consequently, the fragments produced outgrowths of varying activity of both fibroblasts and epithelial cells.

Epithelial cells appeared most frequently as sheets of cells with a

marked capacity for liquefying the substrate. This is due to their proteolytic activity, which occurred only in zones of epithelium. The liquefaction continued as long as the cells existed in culture, as was shown by the repeated liquefaction of new clots. This has been the experience of others working with human breast carcinomas in tissue culture.[3-5] It is not an uncommon activity for epithelium in general, but malignant tissues are considered more active than normal tissue of the same origin.[7,8] Marked liquefaction of the substrate was not observed in cultures of normal breast epithelium. Coman[4] reported less liquefaction from adenomas of the breast after 2 or 3 weeks of cultivation. The marked proteolytic activity of these cultures, therefore, is considered a significant property of the malignant cells.

The epithelial cells rapidly deprive themselves of the plasma clot so that the glass surface becomes their support; therefore, a large part of the time the cells are exposed only to the liquid nutrient. This condition has a direct influence on the behavior of the epithelial outgrowth. The rolling up of the sheet has been cited as one of the results. Separation of sheets of cells into groups and single cells is due, at least in part, to liquefaction. The groups are composed of both closely adherent and loosely connected cells. Some cells become fusiform and very flat and have extended undulating membranes.

Coman[4] attributed this marked separation of malignant epithelial cells to a lack of adhesiveness. He[9] has shown that to separate normal epithelial cells of the lip and cervix more stress was required than for carcinoma cells of the same origin. Coman[4] has suggested that this lack of adhesiveness might be due to a decrease in calcium at the surface of the cells. Carruthers and Suntzeff[10] found a decrease in calcium content in chemically induced squamous cell carcinomas of mice. A reduction in calcium and magnesium in human breast and skin cancers has been shown by Scott.[11] Observations of our cultures indicate that there are variations of this property within the outgrowth, since the cells can roll as a sheet, can remain in compact groups, and can stay attached to the glass for some time.

Epithelial outgrowths from these tumors did not exhibit unlimited proliferation; although a few epithelial outgrowths showed some frequency of mitoses, the majority had only a rare dividing cell or none. This correlates with the lack of appreciable increase in outgrowth after 3 or 4 days' migration, even with regular nutrient changes and addition of new media.

The cytology of cancer cells has been a subject of great interest and study in tissue culture. Although no specific morphologic criterion of

TABLE I
Results of Tissue Cultures

Surgical pathology no.	Cultural behavior	Total days maintained in culture*
	Adenocarcinomas	
29418	Fibroblasts, dense outgrowth Epithelium, sheets of cells	185 60
29739	Fibroblasts, dense outgrowth Epithelium, sheets of cells	90 60
29769	Fibroblasts, dense outgrowth Epithelium, sheets of cells	90 60
31170	Fibroblasts, very dense outgrowth Epithelium, mixed—no definite formations	285 62
31801	Fibroblasts, dense outgrowth Many explants liquefied clot	82
32275	Fibroblasts, dense outgrowth Epithelium, sheets of cells	43 43
32746	Fibroblasts, dense outgrowth Epithelium, sheets of cells	90 53
33590	Fibroblasts, dense outgrowth Epithelium, sheets, cords	180 130
33672	Fibroblasts, dense outgrowth	73
33736	Fibroblasts, dense outgrowth	70
33988	Fibroblasts, dense outgrowth Epithelium, sheets of cells	30 30
53-97	Fibroblasts, dense outgrowth	90
53-215	Fibroblasts, dense outgrowth Epithelium, sheets, tongues	90 30
53-612	Fibroblasts, dense outgrowth Epithelium, sheets, tongues	30 30
53-1217	Fibroblasts, dense outgrowth Epithelium, sparse groups	21 21
29934	Poor outgrowth of fibroblasts	
30072	No outgrowth	
30530	Fibroblasts, fair outgrowth Explants liquefied clot	
32986	Poor outgrowth of fibroblasts Disintegration of explants	
33629	Poor outgrowth of fibroblasts Explants liquefied clot	
33628	Poor outgrowth of fibroblasts	
31147	Poor outgrowth of fibroblasts	
53-977	No outgrowth Explants liquefied clot	
53-1001	No outgrowth Explants liquefied clot	
53-1087	No outgrowth Explants liquefied clot	

TABLE I (Cont'd)

Surgical pathology no.	Cultural behavior	Total days maintained in culture*
	Duct cell carcinomas	
29206	Fibroblasts, dense outgrowth	160
	Epithelium, sheets of cells	120
32291	Fibroblasts, dense outgrowth	30
	Epithelium, sheets of cells	
32454	Fibroblasts, dense outgrowth	60
	Many explants liquefied clot	
32744	Fibroblasts, dense outgrowth	60
	Epithelium, sheets of cells	30
32910	Fibroblasts, fair outgrowth	30
	Epithelium, sheets of cells (few)	30
33692	Fibroblasts, dense outgrowth	130
	Epithelium, sheets of cells	120
33987	Fibroblasts, dense outgrowth	90
34009	Fibroblasts, dense outgrowth	120
	Epithelium, clusters	75
32026	Fibroblasts, dense outgrowth	120
	Epithelium, sheets of cells	90
53-895	Fibroblasts, dense outgrowth	60
	Epithelium, sheets and tongues of cells	30
	Unclassified carcinomas	
28588	Fibroblasts, dense outgrowth	60
29474	Fibroblasts, dense outgrowth	113
	Epithelium, sheets of cells	90
31965	Fibroblasts, dense outgrowth	42
	Epithelium, sheets of cells	42
31970	Fibroblasts, dense outgrowth	60
	Epithelium, sheets of cells	60
28855	Poor outgrowth of fibroblasts	
29744	Poor outgrowth of fibroblasts	
29751	Poor outgrowth of fibroblasts	
29948	Poor outgrowth of fibroblasts	
30147	Poor outgrowth of fibroblasts	

* Cultures of fibroblasts were discontinued when no further evidence of epithelial formations could be observed.

the malignant cell has been discovered, Ludford[12] and Lewis[13] have pointed out that with careful study differences between malignant and non-malignant cells can be recognized. Certain characteristics of malignant cells that have been observed by them and by others have been noted also in the cultures of the present investigation. One of the most constant and striking changes was the increase in nucleolar material, which was usually obvious regardless of the degree of other changes. Haumeder[14] carefully measured the increase in nucleolar

volume in sections of mammary tissue and found that the mean nucleolar area in square μ in sections of human malignant breast tissue was 4.6 while that of normal gland tissue was 2.23. Caspersson and Santesson[15] did ultraviolet microscopic studies of sections of 20 epithelial tumors which included five human breast carcinomas. They reported that their studies demonstrated abnormal activity of the heterochromatin of cancer cells which produces the proteins contained in the nucleolus.

Ludford[12] has stated that there is close correlation between the nucleotide content of the cells and their rate of growth. He observed also that the large volume of nucleolar material may be due to an "increase in the number of nucleolar organizers owing to the polytene condition of the chromosomes." Continuous cultivation of cell strains has shown that malignant cells retain their characteristics throughout the years. None has been known to revert to normal characteristics.[16]

A comparison of 2 tumors of this series *in vivo* and *in vitro* demonstrated some obvious morphologic similarities. The adenocarcinoma represented in Figures 12 and 13 had scattered strands, clumps, and sheets of epithelium, actively infiltrating the fibrous tissue. It was a fairly well differentiated tumor. There were few mitotic figures. The cells (Fig. 14) showed marked variation in nuclear size, shape, and staining characteristics. Tissue cultures of this tumor produced sheets of epithelium (Fig. 15) showing the same cytologic changes, although somewhat more clearly represented, especially with respect to the nucleolar material and cytoplasm. There were no mitotic figures seen in the outgrowths.

Another adenocarcinoma (Fig. 17) showed poorly differentiated, actively infiltrating strands, sheets, and cords of epithelial cells in section. The nuclei (Fig. 18) showed marked variation in size and shape and stained densely. A moderate number of mitotic figures were seen. Rapid outgrowths of sheets (Fig. 16), tongues (Fig. 5), or broad strands of epithelium developed in the tissue cultures. The cytologic appearance was similar to that found in the histologic section which showed about the same frequency of mitotic figures.

Summary

Tissue cultures were made of primary carcinomas of the human female breast. Satisfactory outgrowths were obtained from cultures of adenocarcinomas, duct cell carcinomas, and unclassified carcinomas.

The patterns of outgrowths and their behavior, the types of cells

and their maintenance, and the general cytologic features of the cells have been described.

Comparisons have been made between malignant and normal epithelium.

Preliminary observations of similarities between *in vivo* and *in vitro* cellular structure have been discussed.

We wish to express our appreciation to Mr. Wendell Gillett for preparation of the photomicrographs.

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[*Illustrations follow*]

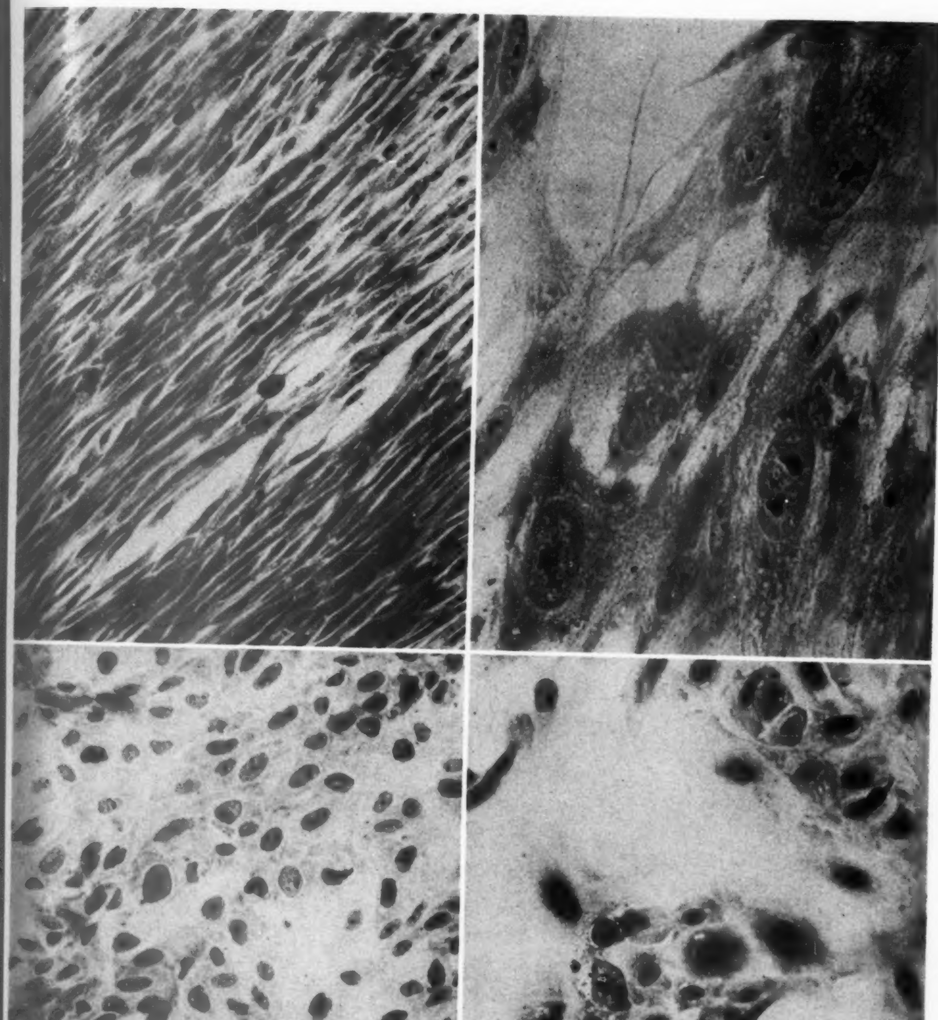
LEGENDS FOR FIGURES

FIG. 1. An outgrowth of fibroblasts from a tissue culture of a specimen of adenocarcinoma of the breast (no. 31170). × 100.

FIG. 2. Nuclei of fibroblasts at a higher magnification, showing variations in shape and amount of nucleolar material. × 600.

FIG. 3. An outgrowth of a sheet of epithelial cells from a tissue culture of an adenocarcinoma of the breast (no. 33590). × 300.

FIG. 4. A separating sheet of epithelial cells. A sister culture of Figure 3. × 300.



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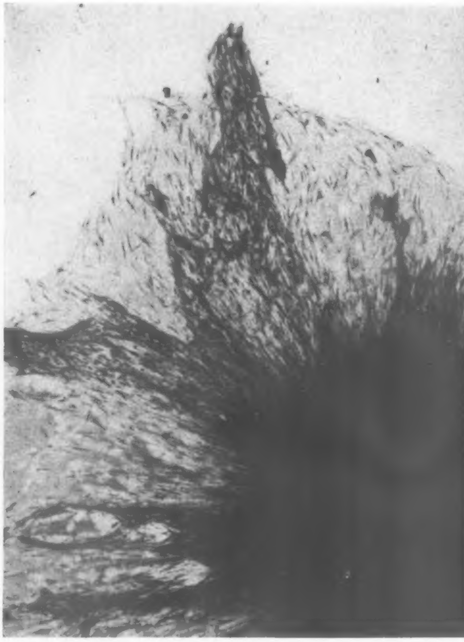

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FIG. 5. Epithelium migrating as cords or tongues of cells in a dense outgrowth of fibroblasts (no. 53-215). × 8.

FIG. 6. Epithelium migrating as cords or tongues of cells with few fibroblasts present (no. 33590). × 100.

FIGS. 7 and 8. Isolated groups of epithelial elements showing also multinucleated cells (no. 29206). × 300.

FIG. 9. Epithelial outgrowth from normal breast tissue (no. 33441). × 300.

FIG. 10. An outgrowth of malignant epithelial cells containing several mitotic figures (no. 33590). × 300.

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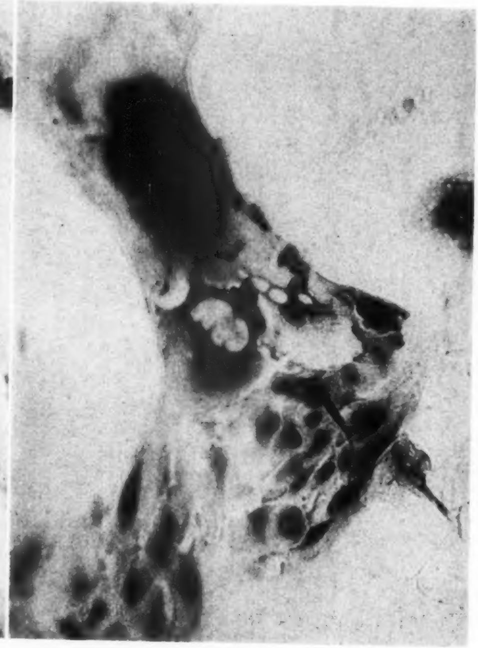

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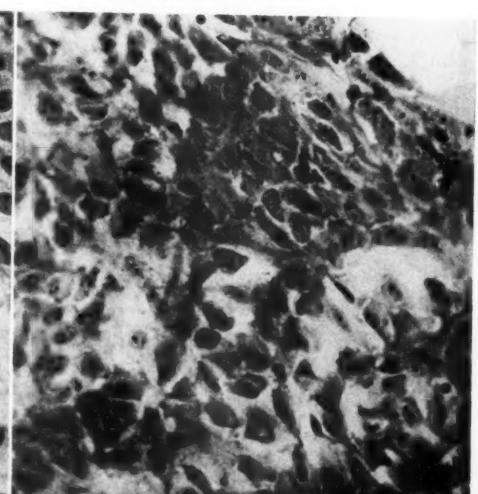

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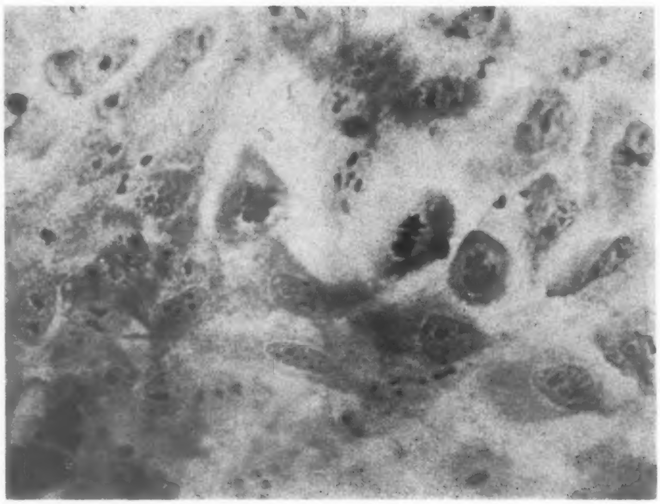

FIG. 11. Three of the mitotic figures in Figure 10. × 600.

FIGS. 12 and 13. Two areas from a histologic section of an adenocarcinoma, showing infiltrating strands and sheets of epithelium (no. 32746). × 65.

FIG. 14. An area of the sheet of epithelial cells in Figure 13 photographed at a higher magnification. × 510.

FIG. 15. An outgrowth of epithelium from a tissue culture of the same tumor represented in Figures 12, 13, and 14. × 255.

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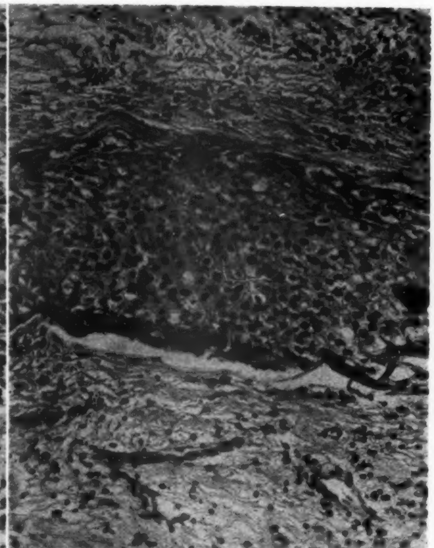
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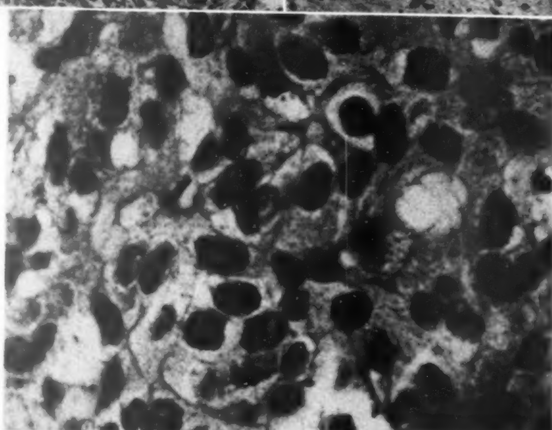
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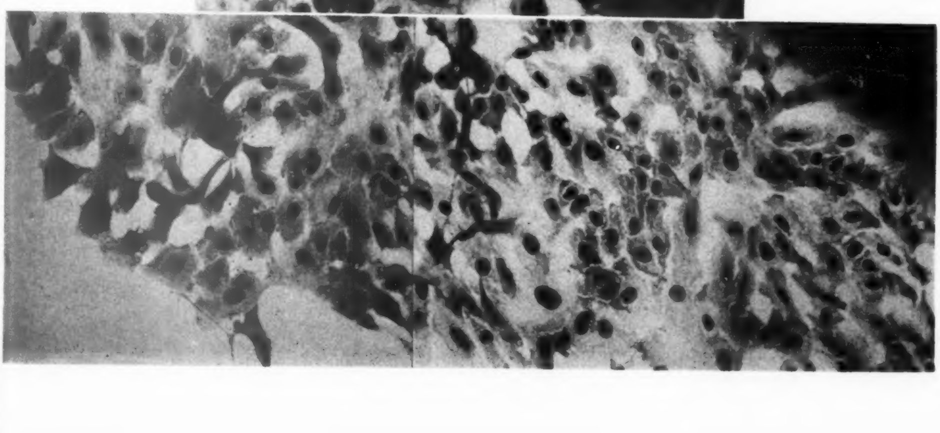
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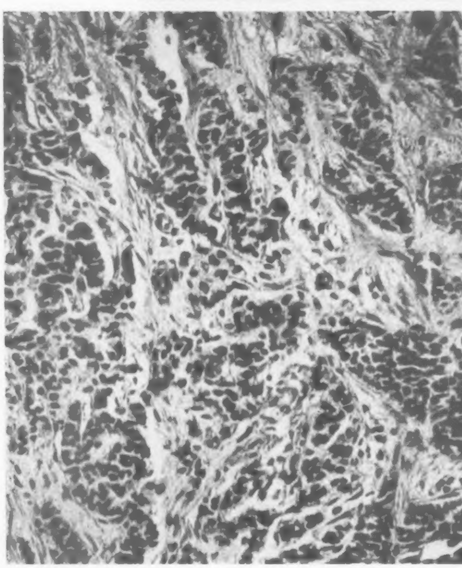

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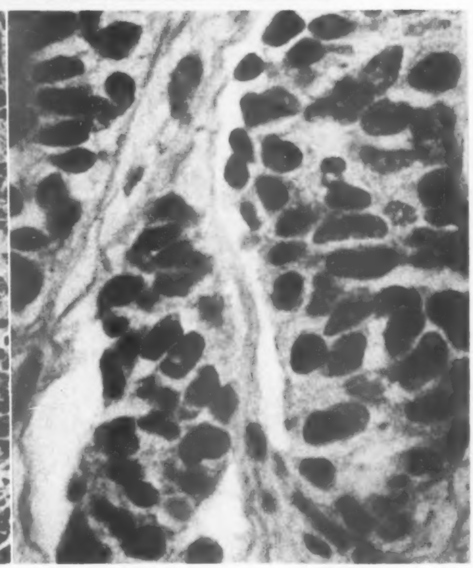

FIG. 16. An outgrowth of epithelium from a tissue culture of the same tumor represented in Figures 17 and 18. × 300.

FIG. 17. Histologic section of an adenocarcinoma of the breast (no. 53-215). × 70.

FIG. 18. An area of epithelium of Figure 17 photographed at a higher magnification. × 550.

FIG. 19. Histologic section of an adenocarcinoma of the breast (no. 33590). × 75.

FIG. 20. Histologic section of a duct cell carcinoma of the breast (no. 53-895). × 75.

FIG. 21. Histologic section of an unclassified carcinoma of the breast (no. 29474). × 75.

FIG. 22. Histologic section of normal breast tissue (no. 33441). × 75.

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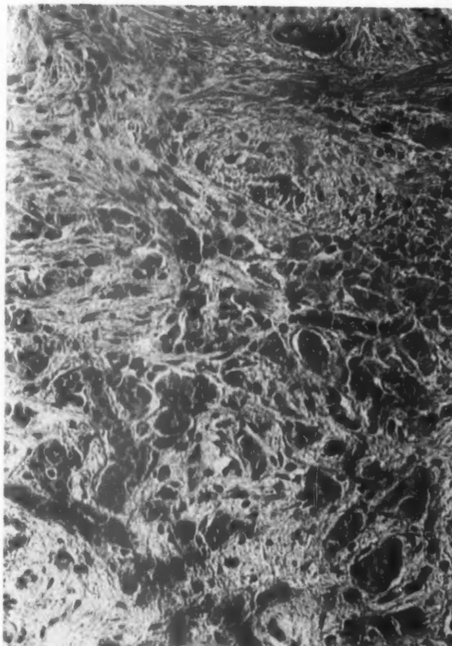

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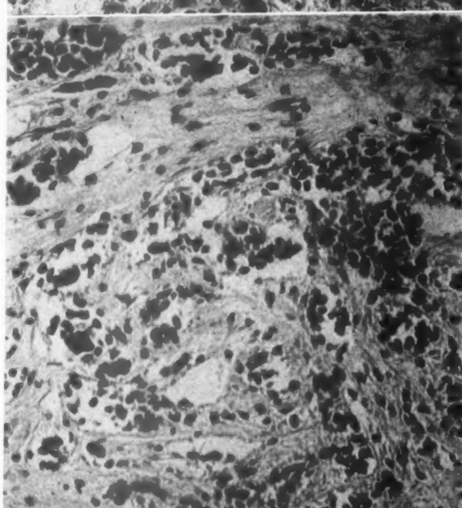

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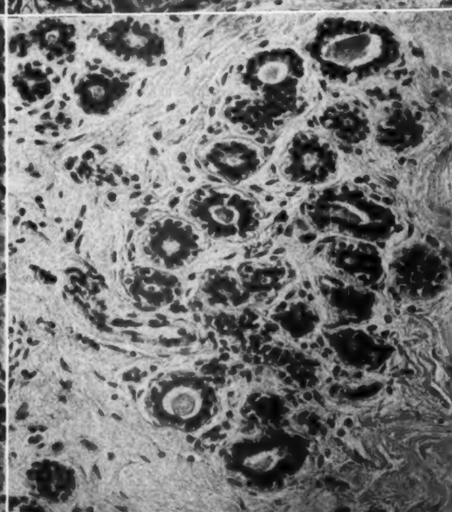

INVOLVEMENT OF INTERNAL MAMMARY LYMPH NODES IN CARCINOMA OF THE BREAST

Studies of the Extended Radical Mastectomy *

John P. Wyatt, M.D.; Everett D. Sugarbaker, M.D., and Mearl F. Stanton, M.D.

From the Department of Pathology, St. Louis University, St. Louis, Mo. (Drs. Wyatt and Stanton), and the Department of Surgery, St. Mary's Hospital, Jefferson City, Mo. (Dr. Sugarbaker)

The Halsted[1]-Meyer[2] radical mastectomy, introduced in 1890, is often cited as the ideal cancer operation, because the primary malignant growth is removed in continuity with chest muscles, axillary lymphatic channels, and nodes; but as far back as 1806,[3] seedling colonies of cancer cells in the internal mammary lymph nodes were recognized at necropsy. Anatomists for years have emphasized the existence of a second important region for lymphogenous dissemination, the internal mammary lymphatic basin.

Within the last 3 years, due to advances in surgical techniques, definitive exploration of the internal mammary lymphatic chain with biopsy study has been employed and an extended radical mastectomy has been accomplished. Histopathologic analysis of this lymphatic chain and of the axillary chain has afforded opportunity to assess the functional significance and relative importance of these two pathways in primary cancer of the female breast.

These studies on homolateral internal mammary lymph node metastasis in association with primary carcinoma of the female breast are based upon anatomical dissections of the tissue removed in 60 extended radical mastectomies.

Material and Methods

The extended radical breast amputations were performed on 60 women with primary cancer of the breast. These cases were drawn from office practice and a private patients' clinic. The extended radical mastectomy comprised amputation of the breast and pectoral muscles with en bloc dissection of the axillary and internal mammary lymph nodes. The latter dissection was performed through a thoracotomy wound. The sternal ends of ribs 2, 3, and 4 were left attached to the surgical specimen. The technical aspects of the operation have been reported elsewhere.[4]

* Presented, in part, at the Fifty-first Annual Meeting of the American Association of Pathologists and Bacteriologists, Philadelphia, April 8, 1954.

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The ages of these women ranged from 27 to 79 years. The surgical operation was performed on women with fixed masses of breast carcinoma as well as clinically early cases. The criteria of operability as outlined by Haagensen and Stout[5] for carcinoma of the breast were followed. The diagnosis of carcinoma from frozen sections was confirmed from permanent sections stained by hematoxylin and eosin.

The surgical specimen was subjected to a detailed examination of the primary mass, nipple, pectoral and intercostal muscles, and axillary and internal mammary lymph nodes. The important topographic markings of significance to the surgical pathologist are shown in Figure 1.

The dissection of the lymph nodes was carried out by members of the Pathology Staff of St. Louis University, and the surgeon (E. S.) gave invaluable assistance in the dissection of the internal mammary lymph node chain. As Stibbe[6] has previously recorded, the internal mammary chain lies deep to the plane of the costal cartilages and is plexiform around the internal mammary blood vessels. Lymph nodes usually are found opposite the first, second, third, and sixth intercostal spaces. Conventional techniques for dissection of the lymph nodes were followed. On the average, 13 to 15 lymph nodes were encountered in the axillary fat and 3 to 5 in the internal mammary chain. These lymph nodes were labelled, embedded, sectioned, and stained with hematoxylin and eosin. Serial sectioning of these nodes was not performed.

As the histologic pattern of the primary carcinoma of the breast was not the primary concern in this particular study, no further reference to cellular structure, either of the primary lesion or its metastases, will be made.

Two case histories, one of Paget's disease of the breast, the other of a persistent epithelial growth within the mammary gland, are incorporated to stress the relationship between the spread of tumor within breast and the temporal pattern of nodal metastases.

Case 1

H. H., a nulliparous, unmarried white female, 47 years old, entered the hospital for a scaly eczema on the nipple of the left breast which had persisted, despite local treatment, for the past 18 months. There had been no discharge from the nipple. Within the past 3 months the patient had noticed cutaneous dimpling of the medial aspect of the breast. Four years earlier the patient had been treated for "congestion" of the left breast. The patient weighed 133 lbs. Pertinent physical findings were confined to the breasts. Three fourths of the areola of the left breast was excoriated and scaly. An irregular, hard mass, 3 cm. in diameter, was attached to the skin of the lower medial side of the areola. A few soft nodes were palpated in the left

axilla. The right breast also showed a mild eczematoid change of the nipple. Roentgenograms of the chest and laboratory findings were essentially normal.

Biopsy of the areolar lesions on both sides and of the mass in the left breast was carried out. The skin of the left areola was invaded by malignant epithelial cells of the type of Paget's cells. Tissue from the right areolar region showed a non-specific inflammatory reaction. An extended radical left mastectomy was done. The breast carcinoma measured 3 by 3 by 3 cm. and was without contiguous extension to the overlying skin. Many large subareolar ducts were occluded by tumor. Examination of the regional nodes revealed that all axillary nodes were free of tumor, but 3 of 5 internal mammary nodes contained tumor cells morphologically similar to those seen in the breast.

Case 2

D. M., a married, multiparous white female, 52 years of age, entered the hospital in February, 1952, with a history of a lump in the left breast of 1 month's duration. There was no history of discharge from the nipple, although multiple cysts of both breasts had been noted previously. On physical examination a firm mass, 1 cm. in diameter, was palpated beneath the skin of the infra-mammary fold on the medial side of the breast. Laboratory findings and roentgenograms of the chest were normal. The clinical diagnosis was fibro-adenoma or mammary carcinoma.

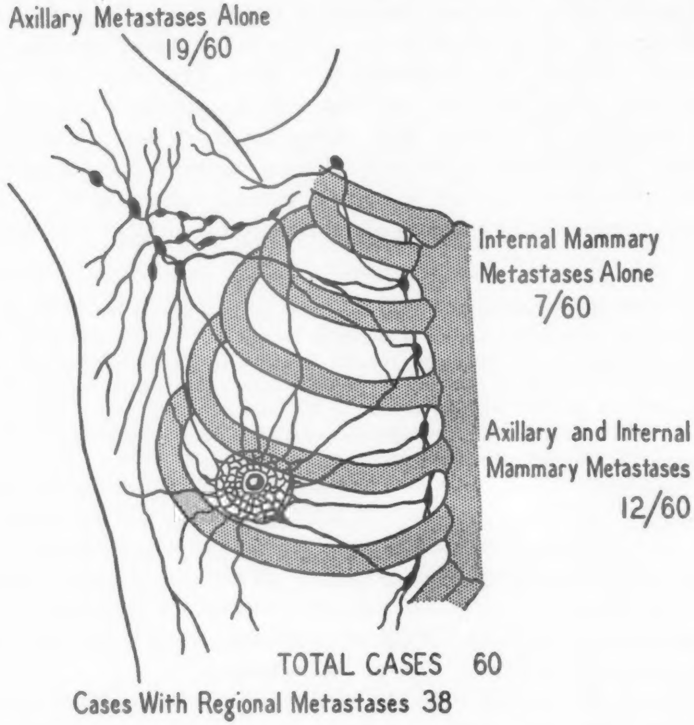
After excision, the tumor was found to be composed of dilated ducts with florid papillary ingrowths of their epithelium. The proliferating ductal cells showed prominent pachychromatic nuclei with abundant eosinophilic cytoplasm and occasional intracellular mucin droplets. Mitotic figures were rare. The basement membranes appeared intact. A diagnosis of multiple intraductal papillomas, transitional cell type, was made and no further surgery was attempted.

The patient returned to the hospital in June, 1953, 16 months after the excisional biopsy, with a recurrent, hard, tender mass beneath the scar. Histologic examination of the lesion showed cells similar to those previously described but with greater variation in structure, discontinuity of basement membranes, and invasion of adjacent parenchyma. The tumor was considered a papillary duct cell carcinoma and an extended radical mastectomy was performed. Examination of the regional nodes showed no evidence of metastatic implants in 18 axillary lymph nodes but all internal mammary nodes contained nests of tumor cells similar to those in the primary lesion. In reviewing the original slides, consultant pathologists concluded that the lesion was, from the beginning, a papillary transitional cell carcinoma.

Discussion

From an examination of Text-figure 1, several basic facts emerge. In the 60 operative cases, 7 instances were noted in which only the

internal mammary lymph node chain contained metastatic tumor; 12 additional examples of metastases to internal mammary lymph nodes were recorded in which tumor deposits also were present in the axillary

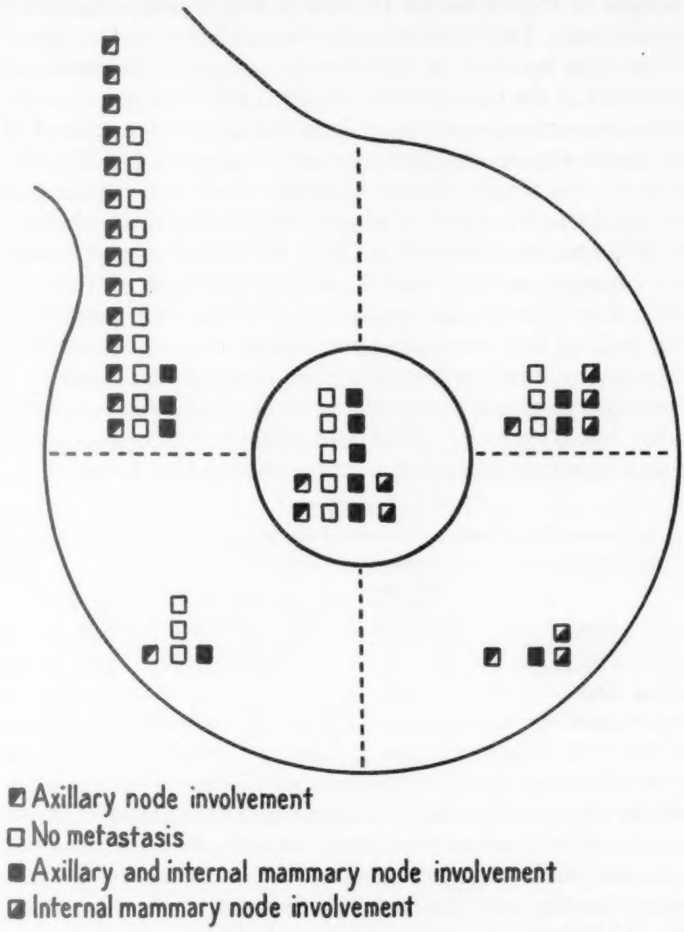


Text-fig. 1. Distribution of regional metastases in carcinoma of female breast.

lymph nodes. A total, then, of 19 cases showed metastatic tumor deposition in the internal mammary lymph node chain.

From a study of Text-figure 2, the positional relationship of the breast cancers to the pattern of lymph node involvement is apparent. Although internal mammary nodal metastases were recorded in association with malignant growths of the outer mammary quadrants, coexistent metastases in the axillary nodes always were present in that group. If outer quadrant tumors are excluded, it is evident that 15 of the 27 medial and subareolar tumors produced internal mammary nodal deposits, and only 12 of these 27 tumors gave axillary metastases. Urban and Baker[7] previously have stressed the frequency of secondary tumor deposits in the internal mammary lymph nodes from

cancer in the medial half of the breast. In their series, 49 per cent of medial tumors had internal mammary node implants. The relatively poor results of radical mastectomy for operable primary breast cancer,



Text-fig. 2. Pattern of lymph node involvement as related to site of primary cancer.

especially when the primary tumor is present in the medial half of the breast, may be due partly to the early involvement of the internal mammary nodal chain which is not extirpated in the course of the usual radical mastectomy procedure.

Case 1 illustrates the surgical significance of internal mammary nodal involvement with infiltration of the skin of the areola. In 2 of

the 60 cases, the earliest clinical manifestation of cancer was Paget's disease of the nipple. In both, internal mammary nodal deposits were present and axillary nodes were free of tumor. Freedom of axillary metastases in Paget's disease is common and is reputed to be a good prognostic sign. This conclusion ignores the importance of lymphatic drainage from the areola to the internal mammary basin and should be re-evaluated in the light of these two documented cases.

From re-examination of case 2, in which the initial treatment of the inner breast cancer was local removal, it appears possible that for months or even longer, cancer cells had lodged in the internal mammary lymphatic depot without reverse flow to the axillary chain. From these observations, which rest solely on location of primary tumor and which probably are unrelated to cellular structure of the primary growth, it is apparent that malignant epithelial new growths in the medial half of the breast may metastasize early and exclusively to the internal mammary nodes and remain there for many months.

The close correlation between our findings and these reported by Handley and Thackray,[8] Urban and Baker,[7] and Andreassen *et al.*[9] (Table I) confirms and strengthens the observations presented in this

TABLE I
Distribution of Nodal Metastases in Clinical Material Reported by Various Authors

Series	Only internal mammary nodes involved	Only axillary nodes involved	Internal mammary and axillary nodes involved	No regional nodes involved	Total
Handley and Thackray[8]	4	42	39	40	125
Urban and Baker[7]	4	9	24	20	57
Andreassen *et al.*[9]	4	28	13	55	100
Wyatt *et al.*	7	19	12	22	60

paper. In essence, these series differed only in the means of investigation and not in the results obtained. In our series, the dissection of the internal mammary lymph nodes was an en bloc investigation; whereas Handley and Thackray,[8] Andreassen *et al.*,[9] and, more recently, McDonald and associates[10] studied the internal mammary lymph nodes through selective biopsies. These surgeons used this procedure for reconnaissance prior to a definitive surgical operation. Urban and Baker[7] limited the dissection of the internal mammary lymph nodes to those cases in which the primary cancer was present in the inner quadrants. In our series, malignant epithelial tumors in all quadrants of the breast were ablated in conjunction with both lymphatic chains. It is our contention that a complete dissection of the

internal mammary chain, irrespective of location of the primary tumor, offers a more objective method of assessing the rôle of the internal mammary lymph nodes in primary carcinoma of the breast.

These studies are presented for their anatomical value only; the therapeutic import of the extended type of radical mastectomy cannot as yet be assessed. But these pathologic studies as well as the related investigations of Urban and Baker,[7] Handley and Thackray,[8] and Andreassen *et al.*[9] reopen the question as to the curative value of the present day classical amputation in approximately one third of patients suffering from cancer of the breast.

An arbitrary clinical formula as to the extent and biologic behavior of breast cancer is frequently used by surgeons. The stages in this classification are listed as from I to IV and are dependent upon local or distant spread of tumor. The ominous grading of tumor spread rests principally upon the clinical finding of fixation of the primary lesion and the presence of axillary metastases. In retrospect, it is probable that many primary malignant new growths of the breast, graded in the past as stage I or II, should, in reality, be considered to be in stage III, due to the unsuspected internal mammary lymph node involvement. Thus the clinical standards of the past prove to be fallacious because they do not take into consideration the results of morphologic analysis of secondary deposits in the internal mammary lymph nodes.

In this series of operable breast cancers approximately 2 of every 3 patients had regional metastases, and in half of those with metastases internal mammary nodes were involved. One of every 5 patients had metastatic implants in both axillary and internal mammary groups. In one of every 10 cases the internal mammary nodes were the sole sites of secondary growth. The anatomical findings derived from this study refute the time-honored concept of metastases in operable cancer of the breast being confined solely to the axillary chain.

From this surprisingly high incidence of metastatic deposits in the lymphatic basin of the internal mammary area, we have at least one explanation to support the dissatisfaction and growing skepticism that has been expressed by McKinnon,[11] McWhirter,[12] and Park and Lees[13] as to the ultimate curative value of the Halstead-Meyer radical mastectomy.

Conclusion

In 60 cases of primary cancer of the female breast, 19 showed secondary growths in the internal mammary lymph nodes and in 7 instances these nodes alone showed metastatic tumor deposits. This

relatively high incidence of internal mammary nodal involvement reaffirms and emphasizes the importance of the anatomical and functional rôle of this lymphatic pathway from the breast.

The primary cancer was located in the medial half of the breast in 13 instances and was subareolar in 14 examples. From these two sites alone metastatic tumor within the internal mammary nodes was found in 15 cases. This tumor-spread along the internal mammary pathway from malignant epithelial growths of the mesial half of the breast is, as far as ultimate biologic behavior is concerned, probably of greater importance than spread along the axillary channels. That the classical Halsted-Meyer operation ignores this lymphatic basin, provides one reason for the present disappointing rate of cure of patients with mammary cancer.

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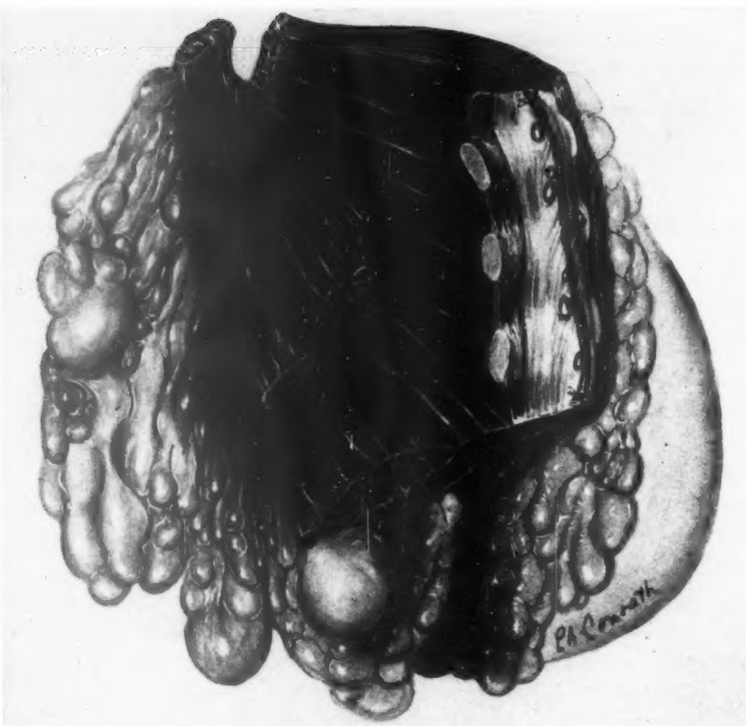

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LEGEND FOR FIGURE

Fig. 1. Tissue removed by the extended radical mastectomy, viewed from the posterior aspect.

THE RÔLE OF OXYHEMOGLOBIN AND ITS DERIVATIVES IN THE PATHOGENESIS OF EXPERIMENTAL HEMOGLOBINURIC NEPHROSIS *

Joseph J. Lalich, M.D.

From the Department of Pathology, University of Wisconsin Medical School, Madison 6, Wis.

Mallory[1] has indicated that renal insufficiency is never progressive in patients recovering from shock in the absence of demonstrable pigment nephropathy. Despite the fact that oxyhemoglobin is essential in the production of hemoglobinuric nephrosis, the mechanism whereby anuria is produced is controversial. It is generally accepted that oxyhemoglobin,[2,3] methemoglobin,[3] and myoglobin[3,4] may not be nephrotoxic in doses of 1 gm. per kg. or less for animals with normal kidneys, normal acid base balance, and adequate reserves of body fluid. Nevertheless, each of these proteins or their derivatives may, on occasion, exert nephrotoxic effects. At such times the glomerular filtration of the protein is associated with one or more of the following: antecedent tubular injury,[5-7] dehydration,[8-10] aciduria,[3,5,7] and phosphaturia.[11,12]

The chemical composition of the pigment casts observed in hemoglobinuric nephrosis, as well as the question of their toxicity, is unsettled. Whether hemoglobinuric nephrosis occurs only when associated with antecedent tubular injury or may develop also following the accumulation of pigment casts in normal tubules requires experimental proof. For example, it has not been conclusively demonstrated whether the anuria is due to insoluble proteinaceous casts which merely plug the tubules, as suggested by Oliver and associates,[13,14] or is the result of epithelial injury by ferrihemate[15,16] or hemosiderin.[17] It is known that extracellular oxyhemoglobin may be converted to hemosiderin by phagocytes[18]; however, factors responsible for hematin formation are unknown.[19] Because the morphologic demonstration of proteinaceous casts or pigments in the renal tubules cannot be accepted as establishing the cause of anuria, additional studies in this connection are highly desirable. The present experiments were undertaken to study the relationship between pigment retention and oliguria, and to determine if some means could be found which would inhibit the development of a protracted oliguria and fatal uremia.

* The investigation was supported by USPHS A-538(C4) Division of Research Grants and Fellowships.

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Method

Many of the associated factors which have been shown to promote fatal hemoglobinuric nephrosis were utilized in this study. Because an acid diet will influence the precipitation of pigment casts in the renal tubules, most of the rabbits were fed ground oats containing 0.25 per cent $CaCl_2$, 0.25 per cent KCl, 1.0 per cent $NaH_2PO_4 \cdot H_2O$, 2.0 per cent brewer's yeast, and 0.2 per cent niacin for at least 2 weeks prior to the intravenous injection of methemoglobin. All rabbits were denied food and water for 2 days prior to methemoglobin injection because reductions of body fluids also predispose to pigment cast formation and pigment retention. In view of the apparent greater nephrotoxic effect of methemoglobin than oxyhemoglobin in rabbits with aciduria, only the former pigment was employed.[3,12,20] The animals were given 1 gm. per kg. of methemoglobin in 3 or 4 doses in an interval of 7 to 8 hours. Methemoglobin solutions of 10 gm. per cent, not older than 3 days, were used.

The methemoglobin was prepared by adding the required volume of 2 per cent potassium ferricyanide to a known volume and concentration of oxyhemoglobin.[20] The volume of 2 per cent potassium ferricyanide to be added was calculated so that 1 mg. of salt was used for every 25 mg. of oxyhemoglobin. A final concentration of 10 gm. per cent of methemoglobin was attained by the addition of a calculated volume of freshly distilled water. Fifty-three New Zealand white rabbits of both sexes, weighing from 1.6 to 3.1 kg., were used in this study. Two experiments were performed.

Experiment A. In experiment A, 26 rabbits received intravenous methemoglobin without subsequent treatment. In order to prove that methemoglobin alone was not nephrotoxic but that its effect was conditioned by the diet, we included 9 animals in experiment A which were fed rabbit pellets* prior to the injection of methemoglobin. Feeding ground oats prior to methemoglobin injection to 17 rabbits established the effect of diet and the expected mortality in the absence of treatment.

Experiment B. In experiment B, 21 rabbits were fed ground oats prior to the injection of methemoglobin. Six other oat-fed rabbits which were not given methemoglobin were treated with BAL (2,3–dimercaptopropanol).

Following the last methemoglobin injection, food and water were

* Master mix rabbit pellets (McMillen Feed Mills, Division of Central Soya Co., Inc., Fort Wayne, Ind.).

given. After an interval of 10 to 15 hours the animals were subjected to one of four types of treatment. Those rabbits which excreted approximately 10 to 25 per cent of injected methemoglobin within 1 to 15 hours were considered unsuitable for treatment and were excluded. Ten per cent BAL diluted 1:10 in sesame oil was administered intramuscularly in doses of 12 mg. per kg. twice daily for 2 days. Buffered (pH 6.5 to 7.1) solutions of 1 per cent disodium calcium versene,* sodium gluconate, and sodium ascorbate were injected intravenously in doses of 25, 50, and 25 mg. per kg. respectively twice daily for 2 days. The urinary excretion, the specific gravity, and the pH were observed in all before and after the injection of methemoglobin or drugs except in 7 pellet-fed and 2 oat-fed rabbits. The non-protein nitrogen was determined by a micro-Kjeldahl method on blood samples obtained at necropsy or on the fifth day after injection.[21] The necropsies were performed shortly after death or by the twelfth day. After gross examination of the organs the kidneys and lungs were weighed. Representative segments of tissue for microscopic examination were fixed in neutral 10 per cent formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. To establish whether both potassium ferrocyanide and hemosiderin were present in the kidneys at the time of death, some of the formalin-fixed kidneys were immersed in 1 per cent HCl for 12 hours. An approximation of the quantity of hemosiderin in the renal tissue was also established by immersing 1 to 3 day-old slices of formalin-fixed kidneys in a solution of 1 per cent potassium ferrocyanide in 5 per cent HCl for 20 minutes.[22] Kidneys fixed in neutral formalin for more than 3 days could not be used because even normal kidneys developed a faint blue color.

Results

Experiment A

Observations on the effect of diet and intravenous injection of methemoglobin without treatment are shown in Table I.

Eight animals fed rabbit pellets before methemoglobin injection all survived. Pigment discoloration and increases in weight of the kidney, as well as increases of plasma non-protein nitrogen, were minimal or absent. These findings indicate that methemoglobin is not toxic and that it is incapable of producing nephrosis. Eight of 13 oat-fed rabbits died from 3 to 9 days following the injection of methemoglobin. These 8 rabbits developed significant elevations of non-protein nitro-

* Bersworth Chemical Co., Framington, Mass.

gen, obvious pigment discoloration, and marked increases in weight of the kidneys. An oat diet supplemented with acid salts when fed

TABLE I

The Influence of Diet on Survival after Injection of 1 gm. per kg. of Methemoglobin

Rabbit no.	Diet	Non-protein nitrogen	Necropsy	Weight		
				Rabbit	Lungs	Kidneys
		mg. %	days	gm.	gm.	gm.
O–19	Pellets	41	8	2661	10.5	16.9
O–26	Pellets	35	8	2087	9.7	13.6
O–27	Pellets	19	10	2614	8.8	15.6
O–28	Pellets	91	10	2150	10.5	15.8
O–34	Pellets	33	13	2822	10.3	16.5
O–35	Pellets	31	13	2666	9.6	18.2
O–42	Pellets	33	9	2775	10.1	17.5
O–49	Pellets	44	8	2922	10.9	15.7
O– 7	Oats	260	6*	1920	7.5	30.8
O– 8	Oats	74	8	2192	8.4	18.9
O– 9	Oats	314	5*	2486	12.2	34.9
O–10	Oats	262	4*	2140	7.1	32.0
O–11	Oats	373	5*	2135	8.9	37.4
O–12	Oats	274	3*	2300	16.6	20.4
O–40	Oats	80	9			
O–46	Oats	89	9			
P– 2	Oats	163	12	2632	8.7	22.8
P– 5	Oats	455	6*			
P– 7	Oats	372	9*	1655	8.5	21.0
P– 8	Oats	435	4*	2435	18.6	28.8

* Rabbit died.

prior to the injection of methemoglobin predisposed to nephrosis. Six untreated rabbits not included in Table I died after the first or second intravenous injection of methemoglobin. Death was usually preceded by cyanosis and precipitous drops in blood pressure. Since 6 or 7 other rabbits failed on repeated occasions to exhibit similar symptoms after methemoglobin injection, it seems reasonable to assume that factors other than methemoglobin are necessary to produce immediate death.

Experiment B

Data obtained from 21 rabbits which were fed oats, injected with methemoglobin, and subsequently subjected to one of four types of treatment are shown in Table II.

Since the toxicity of BAL is dependent upon concentration, it is necessary to establish that the dose which is to be used is not toxic. Six oat-fed animals which were given intramuscular injection of BAL for 2 days are not included in Table II. The plasma non-protein nitrogen concentrations, weight of the kidneys, and the subsequent micro-

scopic examinations of the kidneys all failed to reveal any toxic effects due to BAL. The findings in these 6 rabbits also indicate that ground

TABLE II

The Effect of Treatment Following Injection of 1 gm. per kg. of Methemoglobin in Oat-Fed Rabbits

Rabbit no.	Dose per day†	Non-protein nitrogen	Necropsy	Weight		
				Rabbit	Lungs	Kidneys
	mg./kg.	mg. %	days	gm.	gm.	gm.
O-14	BAL 24	220	2*	2165	21.5	17.8
O-15	BAL 24	211	4*	2112	13.7	39.4
O-16	BAL 24	226	5*	2204	7.6	26.4
O-17	BAL 24	278	5*	2146	13.2	23.7
O-20	Sodium gluconate 100	300	4*		9.6	30.4
O-21	Sodium gluconate 100	320	5*	2400	19.4	26.8
O-22	Sodium gluconate 100	312	6*	2230	12.0	34.3
O-39	Versene‡ 50	238	9*	1970	34.9	29.6
O-41	Versene‡ 50	384	9	2005	8.9	32.2
O-43	Versene‡ 50	328	5*	2477	15.2	30.6
O-45	Versene‡ 50	306	5*	2435	9.4	30.0
O-47	Versene‡ 50	296	2*	3162	16.0	23.5
O-23	Sodium ascorbate 50	47	12	2718	9.9	28.0
O-24	Sodium ascorbate 50	123	12	1910	8.9	19.8
O-25	Sodium ascorbate 50	35	12	1950	8.5	17.5
O-37	Sodium ascorbate 50	302	7*	2467	20.6	37.7
O-38	Sodium ascorbate 50	412	7*	1926	17.5	32.1
P- 1	Sodium ascorbate 50	306	6*	1975	10.2	26.1
P- 3	Sodium ascorbate 50	597	4*	2024	16.3	28.5
P- 4	Sodium ascorbate 50	296	11*	2085	18.6	35.7
P- 5	Sodium ascorbate 50	478	4*	2528	24.7	31.9

* Animal died.

† Rabbits treated for 2 days.

‡ Versene prepared as disodium calcium versene.

oats supplemented with acid salts are not nephrotoxic. Four rabbits injected with methemoglobin and subsequently treated with BAL for

2 days died. The non-protein nitrogen concentration, the increases in weight of the kidneys, and the discoloration of the kidneys by pigment all suggest that treatment with BAL is ineffective. Injection of 1 per cent sodium gluconate into 3 rabbits and 1 per cent disodium calcium versene into 6 other animals was also ineffective. Six of 9 animals treated for 2 days with 1 per cent sodium ascorbate also died. Survival in 3 of 9 rabbits is attributed to individual differences in susceptibility.

Gross Examination of Tissues

In 20 rabbits without nephrosis, including the 12 rabbits not shown in Tables I and II, the weight of the kidneys varied from 11.3 to 19.9 gm. Only 2 of 20 animals had kidneys weighing more than 18 gm. All kidneys were smooth and usually they were pale red. At most, only isolated small (1 to 2 mm.) foci of brown pigment were observed in the kidneys of animals fed pellets and administered methemoglobin. In contrast, both the weight of the kidney and pigment retention were much more pronounced in the 25 rabbits which died. These rabbits were all fed oats supplemented with salt before methemoglobin injection. Pigment retention was moderate or excessive, and the brown discoloration was diffuse. Only one of 25 rabbits which died had a kidney weighing less than 20 gm.

There was also a high incidence of pulmonary edema in the rabbits which died (Tables I and II). In 20 rabbits without nephrosis the weight of the lungs varied from 8.8 to 11.5 gm. Since other pulmonary lesions were excluded by microscopic examination, the increases in weight reflect the pulmonary edema. Fourteen of 25 rabbits which died had lungs weighing more than 13.0 gm. It appears, therefore, that pulmonary edema is a frequent complication of fatal hemoglobinuric nephrosis.

Prussian Blue Reaction

The potassium ferricyanide used for the *in vitro* conversion of oxyhemoglobin to methemoglobin is reduced to potassium ferrocyanide which may be retained in the kidneys providing there is oliguria. In the presence of hemosiderin and potassium ferrocyanide in the renal tubules, only an acid pH is necessary for the formation of Prussian blue. It is possible to demonstrate that both of these compounds were present in the kidneys of 12 rabbits by immersing pieces of formalin-fixed kidney in 1 per cent HCl for 12 hours. The Prussian blue color developed slowly and was not very intense (Fig. 1). In 12 rabbits a crude approximation for hemosiderin alone was attempted by immersing slices of kidney, which had been fixed in formalin for 1 to 3 days, in 1 per cent potassium ferrocyanide in 5 per cent HCl for 20

minutes. Gross observations from this study suggest that the correlation between the presence of hemosiderin and renal failure is poor (Fig. 2), whereas a diffuse retention of the brown pigment (presumably ferrihemate) was invariably associated with oliguria or anuria.

Microscopic Examination

Microscopic changes observed in the kidneys of 25 rabbits which died are in agreement with previous communications on this subject.[7,8,20] Since oliguria or anuria in hemoglobinuric nephrosis has been attributed to the plugging of tubules by casts or to injury of the epithelial cells by toxic derivatives, it seemed desirable to evaluate our data with these questions in mind. It was hoped that such an analysis might add information about the mechanism by which oliguria is produced. Although it was appreciated that microscopic examination of routine kidney sections is not as informative as the detailed analyses of whole nephrons by Oliver and associates,[13,14] it was thought that microscopic evidence of intrarenal obstruction would be of some value. We evaluated the kidneys for compression of glomeruli (Bowman's spaces equal to 25 per cent or more of the glomerular area), for dilated proximal convoluted tubules, and for eosinophilic fluid in Bowman's spaces in 21 rabbits which died. These data and the daily urinary excretion for 4 days are included in Table III.

In the first three columns the level of non-protein nitrogen, the day of death, and the combined weights of the kidney are shown. Diffuse glomerular compression was observed in 6 of 21 rabbits. In 3 other rabbits the compression was segmental. In association with glomerular compression, many dilated proximal convoluted tubules containing eosinophilic material invariably were observed, which is unusual in normal kidneys. In 14 kidneys there was no evidence of either glomerular compression or dilation of the proximal convoluted tubules. Marked dilation of the distal convoluted tubules was observed in 4 rabbits with compressed glomeruli and in 3 others without compression. The presence of some eosinophilic fluid in Bowman's spaces and proximal tubules suggests an alteration in glomerular permeability. Anuria or oliguria was present in 19 of 21 rabbits in which excretion of urine was observed for 4 days after methemoglobin injection. Urine was undoubtedly present in the bladders of some animals during the injection period because the specific gravity of the urine was always lower after the first day. The fact that 5 rabbits urinated only on the first day and some of these failed to excrete any methemoglobin also suggests that urine was present in the bladders during the injection period. There does not seem to be any correlation between glomerular

compression and the total 4-day urine volume. It is of interest, however, that the 2 animals with the greatest total volume of urine did not

TABLE III
Microscopic Observations and Urine Excretion in 21 Rabbits Which Died

Rabbit no.	Highest observed non-protein nitrogen	Died	Combined weights of kidneys	Glomerular compression	Dilated proximal convoluted tubules	Eosinophilic fluid in Bowman's space	Urine volume 1-2-3-4
	mg. %	day	gm.				ml./day
O- 7	260	6	30.8	+	+	+	0-0-0-0
O- 9	314	5	34.9	+	+	+	0-0-0-0
O-10	262	4	32.0	+	+	−	68-0-0-0
O-11	373	5	37.4	−	−	−	95-0-0-0
O-12	274	3	20.4	−	−	−	56-42-0
P- 5	455	6		½+	+	+	94-0-0-0
O-15	211	4	39.4	−	−	−	82-0-11-0
O-16	226	5	26.4	⅛+	+	−	23-0-0-0
O-17	278	5	23.7	−	−	−	118-0-0-196
O-20	300	4	30.4	−	−	−	0-0-0-0
O-21	320	5	26.8	−	−	+	80-0-0-0
O-22	312	6	34.3	¼+	+	+	38-0-0-0
O-39	238	9	29.6	−	−	−	68-0-0-321
O-43	328	5	30.6	−	−	−	0-0-0-0
O-45	306	5	30.0	−	−	−	0-0-0-45
O-37	302	7	37.7	+	+	+	26-0-0-0
O-38	412	7	32.1	−	−	−	0-0-0-0
P- 1	306	6	26.1	+	+	−	39-0-0-135
P- 3	597	4	28.5	+	+	−	3-0-0-22
P- 4	296	11	35.7	−	−	−	0-0-0-0
P- 5	478	4	31.9	−	−	+	0-0-0-41

have glomerular compression. The observation of diffuse or segmental intrarenal obstruction in 9 of 21 rabbits suggests that intrarenal obstruction is a serious and frequent complication, which is undoubtedly exerting some influence in the formation of urine. The occurrence of oliguria and uremia in the absence of intrarenal obstructive nephropathy, as well as the observation of reduction in the ability to concentrate urine, suggests that tubular injury also is produced.

DISCUSSION

In confirmation of previous reports, it was possible to show that methemoglobin toxicity is influenced appreciably by the pH of the urine.[3,20] The oliguria and uremia were always associated with the retention of protein casts, a brownish discoloration of the cortex, and appreciable increases in weight of the kidney. The experimental findings in this study, therefore, are in agreement with Mallory's[1] observation in man. It is not possible to state from these studies whether the increased weight of the kidney is due to the retention of protein

casts, interstitial edema, or both. In support of Oliver's[13,14] hypothesis it was possible to demonstrate some intrarenal tubular obstruction in almost half of the rabbits which died. The presence of uremia, however, in the absence of intrarenal obstruction suggests that still other factors are exerting an effect on tubular function.

The gross and microscopic changes observed in the lungs of animals dead from uremia indicate that pulmonary edema is a frequent and serious complication in fatal hemoglobinuric nephrosis. This is the first time that this observation has been made on a large number of animals. This finding is in agreement with pulmonary changes which are frequently encountered in man. Whether the pulmonary edema is due to cardiac decompensation or is secondary to alterations in pulmonary capillary permeability is not evident from these studies. Appreciation that pulmonary edema is a common and serious complication in hemoglobinuric nephrosis should make one cautious in prescribing intravenous fluids in these cases.

That the tubular injury in hemoglobinuric nephrosis may be caused by the presence of toxic material has been suggested by many authors.[4,15,17,22] It is known that antecedent tubular injury predisposes to the accumulation of casts and uremia.[5–7] An objective in this study was to establish whether the retained pigments or the proteinaceous casts are capable of injuring normal renal tubules. The observations which have been made indicate that hematin, hemosiderin, or the protein casts are able to produce injury to healthy tubules. Rabbits which were fed oats supplemented with acid salts and which did not receive methemoglobin failed to manifest any functional renal disturbances or to develop nephrosis. Animals which were able to excrete from 15 to 25 per cent of the injected methemoglobin within 1 to 15 hours always survived. These rabbits at necropsy had kidneys in which the hematin retention was absent or minimal and the renal tubules were normal. Lastly, only those rabbits died in uremia which retained moderate to excessive quantities of brown pigment in their kidneys.

The chemical composition of the toxic derivatives in the renal tubules is still a matter of conjecture. In contradiction to previous suggestions it appears unlikely that either methemoglobin[9] or myoglobin[22] exerts any toxic effect without prior degradation. That denaturation occurs is evident because hemosiderin has been demonstrated in the epithelial cells of the proximal convoluted tubules.[23-25] It was possible to demonstrate that both hemosiderin and hematin were still present in many of the kidneys up to 12 days after injection. In view of the fact that two organic iron compounds may accumulate

in the renal tubules after methemoglobin injection, attempts were made to determine whether the tubular dysfunction is related to hematin, to hemosiderin, or to both. Because hematin possesses a brown color which is not affected by 1 per cent potassium ferrocyanide in 5 per cent HCl, such a differentiation is possible.[26]

Even though our data is semiquantitative, some remarks pertaining to the relative toxicity of hematin and hemosiderin in experimental hemoglobinuric nephrosis are possible from our observations. Gross observations made on formalin-fixed kidney slices which were immersed in 1 per cent potassium ferrocyanide in 5 per cent HCl suggest that hematin may be more toxic. This opinion is based on evidence for minimal or no hematin in the kidneys of rabbits which survived, whereas rabbits which died always had moderate to excessive concentrations of hematin in their kidneys. Fluctuation in hemosiderin concentration appears to be independent of retained hematin. Finally, some of the rabbits which survived appeared to have retained as much hemosiderin as other animals which died.

The treatment employed in this study was designed either to inactivate ferric iron with BAL or versene or effect a redistribution of the iron in the body by increasing its solubility with gluconate or ascorbate. Neither of these measures proved effective in combating the development of oliguria, uremia, and death. Inability to modify the course of this disease by treatment also suggests that other factors besides hemosiderin are exerting toxic effects on the renal tubules. Our analyses for pigments were semiquantitative at best, and the deductions based on these observations are, therefore, only suggestive. The toxic effects of these pigments can be resolved only by studying the relationship between the quantity and type of pigment which is retained in the kidneys and the degree of renal dysfunction which develops.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Segments of 1 to 3-day-old formalin-fixed kidneys from rabbits nos. O-20 to O-25 (Table II) were immersed for 12 hours in 1 per cent HCl solution. Kidneys retaining both hemosiderin and potassium ferrocyanide developed a faint blue color. Rabbits 20 to 22 died, whereas nos. 23 to 25 survived. Brown pigment discoloration is more intense in the kidneys of rabbits which died. The brown pigment is evident as streaking in the cortex and a diffuse discoloration of the medulla. The rabbits which survived have much less brown pigment in the cortex, and the medullae are not discolored.

FIG. 2. Other slices of kidneys from the same rabbits as those from which Figure 1 were taken, which were immersed in 1 per cent potassium ferrocyanide in 5 per cent HCl for 20 minutes, developed a more intense Prussian blue. It is interesting to observe that the rabbits which survived have as much or more hemosiderin than the rabbits which died. In both groups the hemosiderin collected only in the cortex. The differences between these two groups of preparations are a greater concentration of hemosiderin in the cortex and the diffuse discoloration of the medulla by brown pigment. These findings show a better positive correlation between the retention of brown pigment and renal failure. The findings suggest that the brown pigment is more nephrotoxic than hemosiderin.

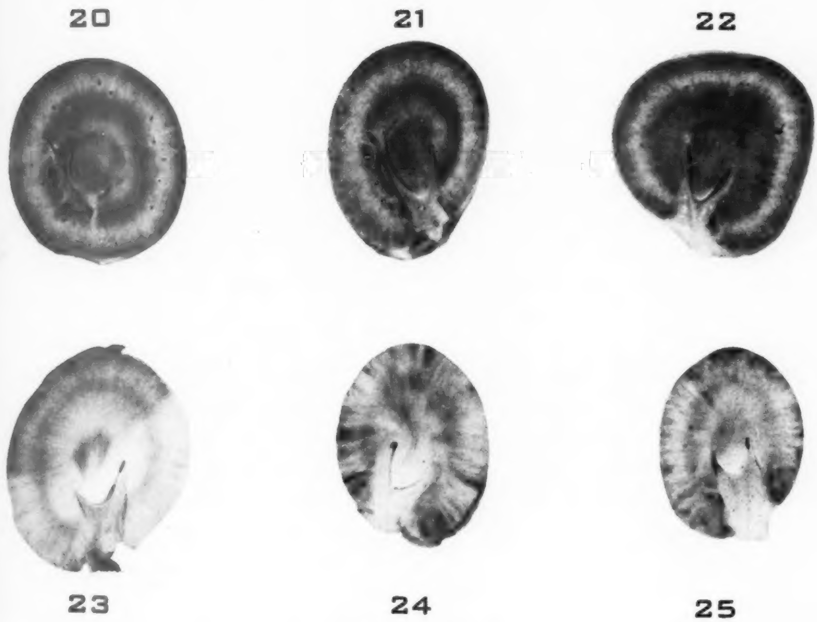

Figure 1

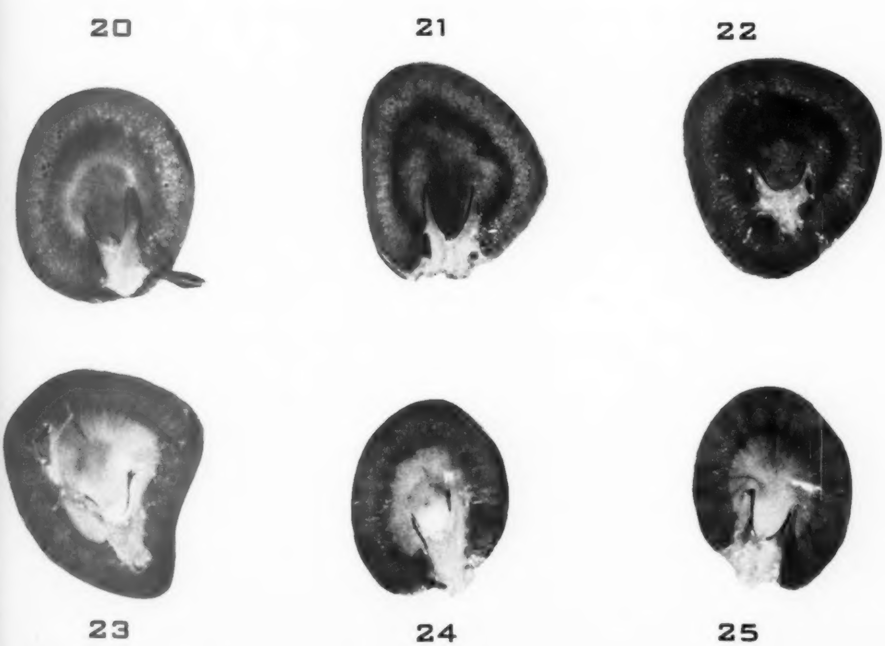

Figure 2

HISTOPATHOLOGY OF EXPERIMENTAL GLOMERULAR LESIONS SIMULATING HUMAN DIABETIC GLOMERULOSCLEROSIS *

J. M. B. BLOODWORTH, JR., M.D., and GEORGE J. HAMWI, M.D.

From the Department of Pathology (Dr. Bloodworth) and the Division of Endocrinology of the Department of Medicine (Dr. Hamwi), College of Medicine, Ohio State University, Columbus 10, Ohio

While studying the effect of cortisone on experimental glomerulonephritis, Rich *et al.*[1] observed aneurysmal dilation of glomerular capillaries associated with the development of nodular lesions resembling those described by Kimmelstiel and Wilson[2] in human diabetic patients. Friedenwald[3] and Becker[4] produced similar lesions by cortisone administration and showed that retinal micro-aneurysms also would develop if the animals were pretreated with alloxan. They called attention to the frequent association of retinal and glomerular lesions in the human diabetic patient.

Because of the great difficulty in evaluation of chronic degenerative changes in the human with diabetes, we have studied these cortisone-produced lesions with the hope that they may present an experimental approach to this problem.

METHODS

Adult rabbits of both sexes were given 5 or 10 mg. of cortisone daily in a single intramuscular injection for a minimum period of 21 days. In some cases the rabbits were pretreated with alloxan, 100 mg. per kg. intravenously. The tissues were formalin-fixed and examined by multiple staining techniques, serial sections, and phase microscopy. Formalin-fixed material from cases of human diabetic glomerulosclerosis was obtained for comparative studies from the collections of the Department of Pathology.

RESULTS

Experimental

The administration of cortisone to rabbits produced glycosuria which varied from 1 to 4 plus and reached a maximum intensity in the second or third week of treatment, following which it gradually disappeared. The alloxan-cortisone group showed glycosuria throughout the course of the experiment with a peak output of glucose in the second or third week. In all other aspects the two groups were similar. Albuminuria appeared during the second or third week—just as the

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glycosuria was falling—gradually rose to a level of 75 to 100 mg. per 100 cc., and was maintained at that level throughout the course of cortisone therapy. The blood urea nitrogen, cholesterol, and plasma proteins showed no significant trend.

Experimental lesions appeared in the glomeruli of the rabbits concurrently with the onset of albuminuria. They were first noted on the 13th day of cortisone therapy and were present in all animals by the 21st day. Maximum frequency was reached between the 21st and 40th days. If the cortisone was continued beyond this time, the experimental lesions gradually decreased in number. However, occasional examples were found in an animal treated for 130 days. If cortisone was gradually stopped after experimental lesions had developed (confirmed by renal biopsy), they would completely disappear in from 4 to 6 weeks.

The experimental lesions were present in from 5 to 10 per cent of the glomeruli; only rarely were two lesions present in the same glomerulus. They were round or oval structures, rarely crescentic, and varied from 30 to 100 μ in diameter. The typical lesion was a homogeneous eosinophilic mass (Fig. 2). In some instances it was vacuolated (Fig. 3), and occasionally a capillary was filled with a thin, foamy, pink precipitate (Fig. 4). Many transitional stages were present. All forms gave the same staining reactions and appeared to be stages of the same process. These lesions have been grouped together and will be referred to as the experimental lesions. Special stains and serial sections indicated that these lesions were predominantly within the lumina of dilated glomerular capillaries. Some lesions only partially occluded the lumen, leaving at one edge a small residual channel filled with red blood cells. Other lesions showed trapped red blood cells within the eosinophilic mass (Fig. 5).

Other histologic changes were noted during the early phase of the study (21 to 50 days of cortisone therapy). An occasional crescentic mass of eosinophilic material was found in Bowman's space, forming a cap over a loop of the glomerular tuft (Fig. 6). These were similar morphologically to the "fibrin caps" seen in the human kidney of diabetic glomerulosclerosis. Also seen were capillary dilation (Fig. 7) and an increase in the number and size of the nuclei of the glomerular epithelial cells. Occasionally eosinophilic material was found in the intercapillary space adjacent to one of the experimental lesions (Figs. 8 and 9). Vascular changes were negligible.

Long-term studies have not yet been completed; however, several animals have been treated for 4 months. The kidneys of these animals

showed arteriolar sclerosis, focal pyelonephritis, and a diffuse thickening of the intercapillary septa (Figs. 13, 14, and 15) quite similar in nature to the changes described in the human diabetic patient by Laipply *et al.*[5] as "focal fibrosis," by Bell[6] as "diffuse intercapillary glomerulosclerosis," and by others[7] as "axial thickening." Occasional glomeruli showed nodular, almost homogeneous masses of dull eosinophilic material with peripheral capillary loops closely resembling the nodular lesions of human diabetic glomerulosclerosis.

Human Material

Several human kidneys which showed the typical nodular lesions described by Kimmelstiel and Wilson[2] were selected for comparative study. Moderate or severe arteriolar sclerosis was present in all. Diffuse thickening of the intercapillary septa (axial thickening) often blended with the typical nodular lesions. Several other glomerular changes, which appeared to represent variations of the same basic process, were observed regularly. These changes have been studied carefully because of their striking similarity to the experimental lesions. They were first described by Fahr[8] and have been evaluated subsequently under such terms as glomerular fibrinoid caps,[9] hyaline-fibrinoid lesions,[10] fibrin cap, capsular drop,[11] and exudative lesions[12,13] (Figs. 16 and 17). Hereafter we shall group these changes under the non-specific term of exudative lesions. They were composed of an eosinophilic material which in solid masses closely resembled hyalin but gave the staining reactions of fibrinoid as outlined by Altshuler and Angevine.[14] Often the lesions were composed of a foamy pink precipitate or a vacuolated eosinophilic mass. A single lesion frequently showed a transition from a solid mass to a vacuolated or foamy lesion. In most cases these lesions appeared to be located in the lumina of glomerular capillaries. Often they appeared only on the peripheral side of a capillary loop with a patent lumen centrally. In these cases it was difficult to determine whether they were thrombi which only partially occluded the lumen or, as Allen[15] suggested, represented deposition of a mucopolysaccharide between the layers of a split capillary basement membrane. Occasionally this eosinophilic material seemed to occupy the intercapillary space. Crescent-shaped masses of it sometimes were found within Bowman's space, covering the greater curvature of a glomerular capillary loop. These are the so-called fibrin caps. Deposition of a similar eosinophilic material between the basement membrane and the epithelial cells of Bowman's capsule gives rise to the "capsular drop."

Special Stains

Special Basement Membrane Stain of Kimmelstiel and Wilson.[2] The human nodular lesions stained deep blue and frequently showed lamination. The experimental and human exudative lesions showed similar staining reactions, varying from a thin blue precipitate to a solid deep-blue homogeneous mass. When the sections were more heavily stained with orange G, these lesions often appeared metachromatic with an orange hue. The diffuse thickening of the intercapillary septa (axial thickening) was well shown by this stain. The thickened septa were dark blue and often appeared continuous with the typical nodular lesions. The Mallory-Heidenhain azan and similar modifications gave a more brilliant differentiation but a similar color pattern.

Lipid Stain, Sudan Black B Impregnation of Frozen Sections of Formalin-Fixed Tissues. The human nodular lesions contained a very small quantity of lipid dispersed to give a fine stippling. The human exudative lesions contained varying quantities of lipid, usually dispersed as small vacuoles. Occasionally an entire lesion was deeply stained. About half of the experimental lesions showed a smaller quantity of lipid dispersed as fine vacuoles. The usual tubular lipid was present.

Examination by Polarized Light for Refractile Material. Human nodular lesions were negative. Both experimental and human exudative lesions showed an occasional focus of doubly refractile material which appeared to be part of the lipid component.

Crystal Violet Stain for Amyloid. The human nodular lesions were blue with a slight purple tinge which was distinctly different from the background blue but did not approach the bright lavender of amyloid. Other lesions were negative.

Gram Stain. The human nodular lesions showed a non-specific, pale red-purple, background color. The experimental and the human exudative lesions stained the rich purple color of fibrin.

Silver Impregnation Method of Gridley.[16] The human nodular lesions were gray-tan and showed black laminations. The human exudative and experimental lesions showed a granular gray precipitate with occasional black fibrils resembling the laminations of the nodular lesions. Thickened intercapillary septa revealed a granular black precipitate as well as occasional black fibrils (Fig. 11).

Phosphotungstic Acid Hematoxylin Stain. The human nodular lesions were homogeneous and tan-brown. The experimental and human exudative lesions had an irregular dark-blue fibrillar pattern superimposed on a solid tan-brown background.

Van Gieson's Stain. The human nodular lesions were homogeneous and dull pink; the human exudative and the experimental lesions, varying shades of tan-brown.

Glycogen Stain, Best Carmine Stain on Ethyl-Alcohol-Fixed Tissues. The experimental lesions were negative. Human material was not available for study; however, previous studies have given negative results.[17,18]

Periodic Acid-Schiff-Glycerin Hematoxylin Stain.[19] The human nodular lesions were positive. Although the intensity of the red color varied, in most cases it was slightly less than that of the basement membranes. The human exudative lesions were more often brilliant red with an intensity similar to that of the basement membranes. All structures of the rabbit kidney stained less intensely than those of the human material. The experimental lesions, however, stained with an intensity similar to that of the basement membranes (Fig. 12). When glycerin hematoxylin was replaced by fast green as a counterstain, the human nodular lesions were blue-purple and showed laminations; the human exudative lesions and the experimental lesions were deep blue.

Discussion

A practical method for the experimental production of diabetic glomerulosclerosis in laboratory animals would greatly facilitate the study of this condition. Previous attempts to produce the typical nodular lesions have been predominantly unsuccessful. Lukens and Dohan[20] produced nodular glomerular lesions in a dog made diabetic by the administration of anterior pituitary extract, but only after 5 years of treatment. Mann and Goddard[21] reported glomerular changes in rats 50 to 90 days after production of alloxan diabetes. They described the presence of hyalin-like balls in some instances. On the other hand, Day and Becker[22] were unable to produce diabetic glomerulosclerosis in rabbits by alloxan alone. Foglia, Mancini, and Cardeza[23] found diffuse glomerulosclerosis in rats made diabetic by subtotal pancreatectomy, but did not mention nodular changes.

The cortisone-produced lesions were first compared with the nodular changes of diabetic glomerulosclerosis because of a superficial morphologic similarity. However, numerous facts support this association and indicate that there may be more than a casual morphologic relationship.

Adrenal cortical function occupies a prominent position in the normal metabolism of carbohydrate. The administration of large doses of cortisone or related steroids produces changes in the body metabolism which closely, if not actually, simulate diabetes mellitus.[24,25] Secondly, adrenal cortical hyperplasia often is present in patients with poorly

controlled diabetes. It is this group of poorly controlled diabetic patients who most often develop degenerative changes. Finally, hypoglycemia and acidosis have been shown to increase the urinary output of 11-oxysteroids (the glucocorticoids).[26]

The onset of the Kimmelstiel-Wilson syndrome is associated with amelioration of the glycosuria and the appearance of albuminuria. It is interesting to note that the experimental animals showed a similar excretion pattern and that the lesions appeared shortly after these changes.

The dilation of the glomerular capillaries and increase in the nuclei of glomerular epithelial cells seen in the experimental animals have been reported also in human diabetic glomerulosclerosis.[6,27] The dilated glomerular capillaries have been compared to the retinal microaneurysms so common in diabetes mellitus, especially after the development of the Kimmelstiel-Wilson syndrome.

Both morphologically and by staining reactions the experimental lesions show a striking similarity to the human exudative lesions. The short period needed for the development of these lesions in the experimental animal as well as the variability in the structure and staining reactions of the human exudative lesions suggests a recent vintage and progressive nature. The human exudative lesions are more frequent in severe diabetic glomerulosclerosis with advanced arteriolar sclerosis and a rapidly progressive clinical course. Diligent search, however, will reveal occasional exudative lesions in almost all kidneys showing the nodular lesion. It is interesting to speculate that the exudative lesions may represent an early stage in the pathogenesis of typical nodular changes.

Previous studies of the chemical nature of the human nodular lesions have been limited. The nodules do not stain for amyloid or glycogen.[17] Allen[15] demonstrated that they were very resistant to tryptic digestion. McManus[18] showed that the "hyalin" of the nodule is not removed by diastase but is removed from acetone-fixed material by pectinase. His studies indicate that it reacts like a mucopolysaccharide with a low protein content in the same manner as do the tubular basement membrane, the hyalin of arteriosclerosis, mucin, or the ground substance of cartilage. Its reaction differs from the glomerular basement membrane or the hyaline deposits seen in glomerular ischemia.

Our studies indicate that the experimental and human exudative lesions are similar in nature and, as the nodular lesions, have a high mucopolysaccharide content. An abnormality in mucopolysaccharide metabolism has been suggested previously by Friedenwald[3] and

Becker,[4] who showed that diabetic retinal micro-aneurysms are associated with an abnormal deposition of mucopolysaccharide in the vessel wall. The presence of abnormal mucopolysaccharide metabolism in the diabetic patient is further supported by the report of an increased glucosamine content of the blood.[28,29] Glucosamine, a prominent component of many mucopolysaccharides, has been shown to follow closely the blood glucose level.

Lipid is another prominent component of the experimental and human exudative lesions. Serum cholesterol is commonly elevated in diabetes mellitus and several previous papers have stressed the increased quantity of lipid in the glomeruli at necropsy.[10,12,30,31] Recently, esterified fatty acids have been shown to parallel the hyperglycemia in diabetic patients.[32]

From our experimental work it appears that the administration of cortisone produces a diabetes-like state followed by albuminuria, aneurysmal dilation of glomerular capillaries, and the formation of eosinophilic thrombi in these dilated capillary loops. The thrombi have a high mucopolysaccharide content and contain variable quantities of lipid. Following thrombosis there is apparently a diffusion of similar eosinophilic material into the intercapillary space or the adjacent lumen of Bowman's capsule. It is also possible that this material may be deposited between layers of the basement membrane. With continued administration of cortisone the experimental lesions were replaced by a diffuse intercapillary thickening. We have stated previously that the experimental lesions disappeared. However, we still are investigating the possibility that the thickening represents a progression of the experimental lesions. It seems more likely that the thickening is an associated phenomenon possibly stimulated by the vascular changes induced by capillary plugging or secondary to diffusion of eosinophilic material into the intercapillary spaces during the early phase of the experiment. Finally one must consider the possibility that the diffuse thickening is completely unrelated to the experimental lesions. Additional long-term studies are under way to determine whether the experimental lesions may in time progress to a typical nodular form of diabetic glomerulosclerosis.

Summary

Lesions were produced in rabbit glomeruli by the administration of cortisone. Histologic study showed that these lesions were thrombi composed of mucopolysaccharide and lipid within dilated capillaries. The experimental lesions are similar to human "exudative lesions"

frequently associated with the typical nodular lesions of human diabetic glomerulosclerosis. Interrelationships between "steroid diabetes," the experimental lesions, and diabetic glomerulosclerosis are discussed.

We wish to express appreciation to Mr. R. A. Flumerfelt and his staff for the preparation of the histologic sections and many special stains; to Mr. G. R. Millard for the photography; to Mr. D. H. Shoffstall and Mr. H. C. Spencer, students in Veterinary Medicine, for technical assistance and care of the animals; and to Merck & Co. for supplying the cortisone used in this study. We also wish to acknowledge the financial assistance given this project by the Comly-Coleman Fund and the Institute of Nutrition of Ohio State University.

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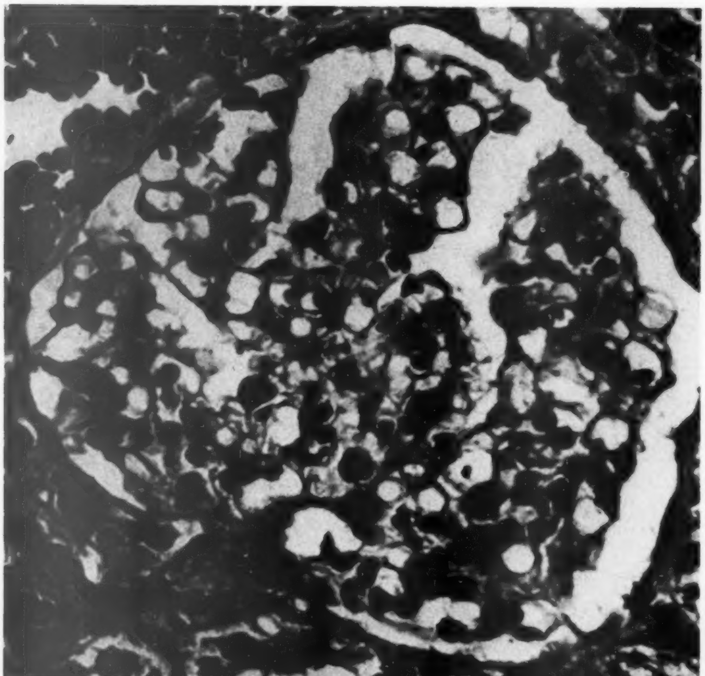
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Rabbit. Normal glomerulus. Of note are the size of the capillary lumina and the delicate nature of the intercapillary septa. Hematoxylin and eosin stain. × 810.

FIG. 2. Rabbit. Experimental lesion of the hyaline type. Hematoxylin and eosin stain. × 835.



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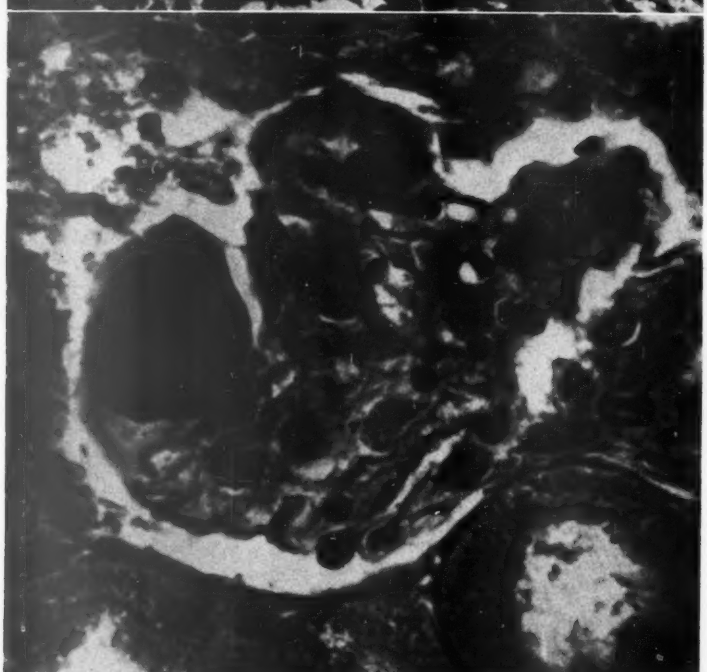

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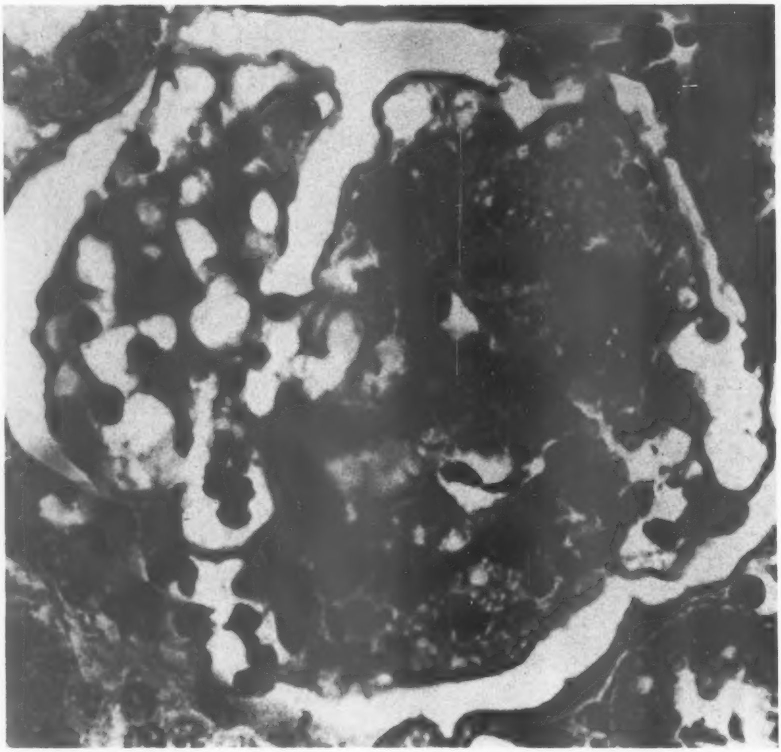
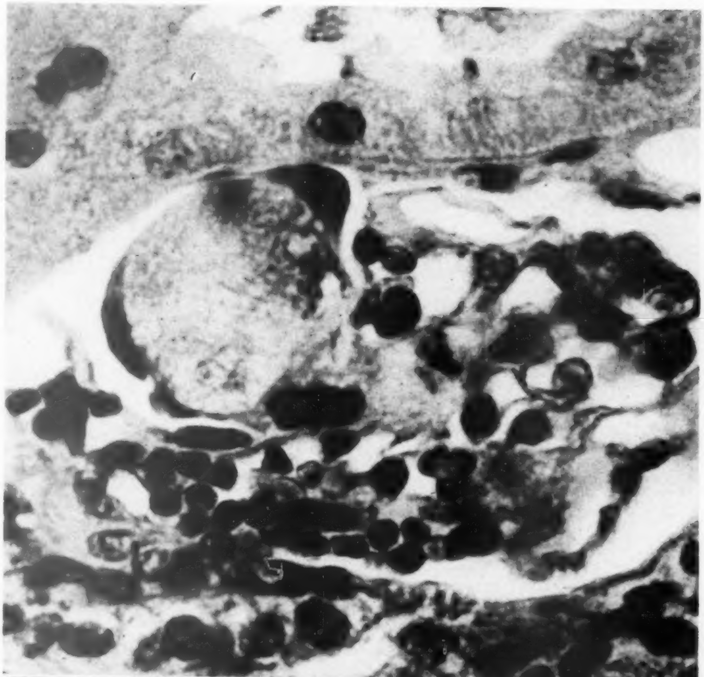


FIG. 3. Rabbit. Experimental lesion of the vacuolated type. There are two lesions; of note are the residual lumen in one and the trapped blood cells. Hematoxylin and eosin stain. × 925.

FIG. 4. Rabbit. Experimental lesion of the foamy type. Hematoxylin and eosin stain. × 945.

FIG. 5. Rabbit. Experimental lesion with trapped red blood cells. Hematoxylin and eosin stain. × 1250.



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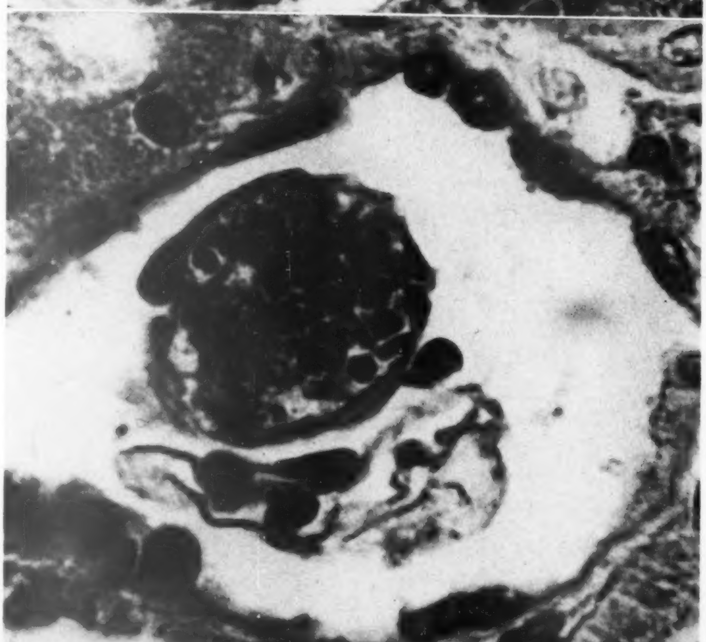

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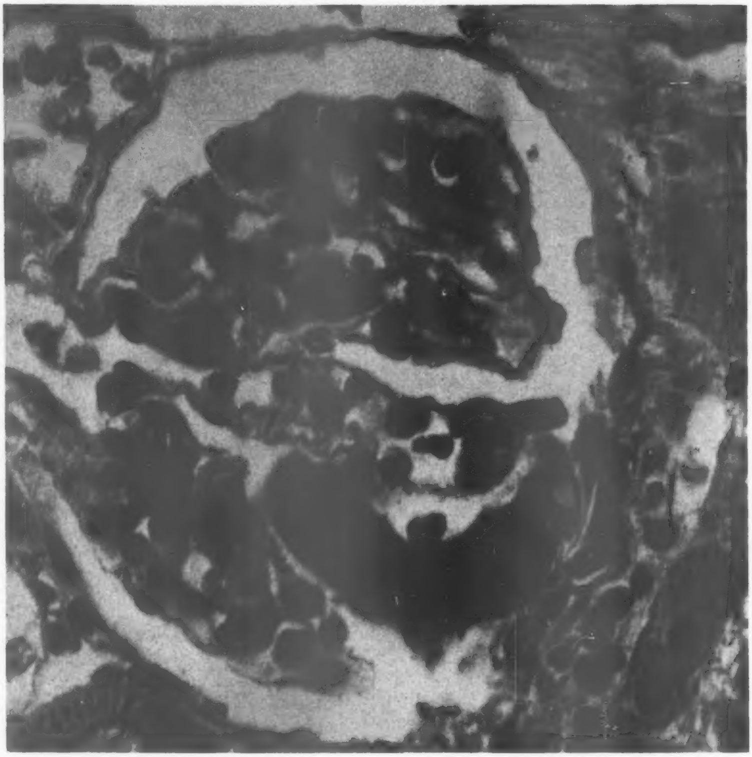
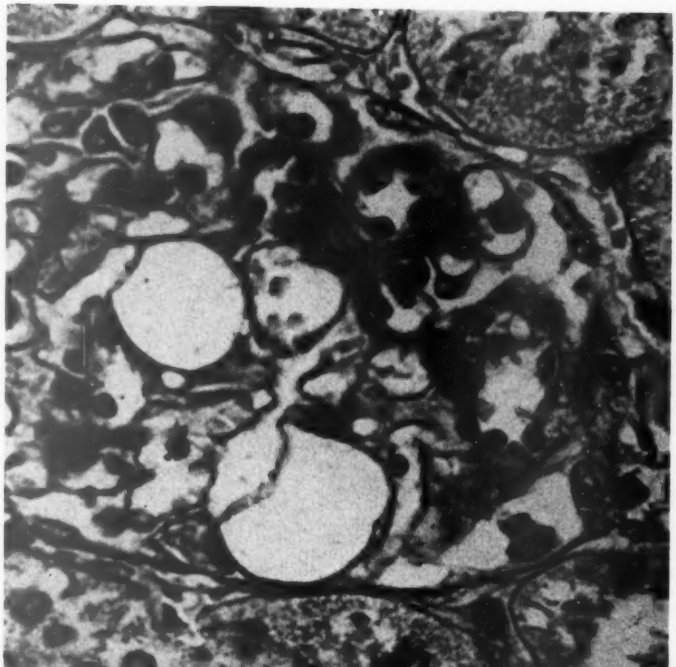


FIG. 6. Rabbit. "Fibrin cap." A mass of eosinophilic material completely free in the lumen of Bowman's space. Hematoxylin and eosin stain. × 900.

FIG. 7. Rabbit. Dilated capillaries ("micro-aneurysms"). For comparison with Figure 1. Periodic acid-Schiff-glycerin hematoxylin stain. × 835.

FIG. 8. Rabbit. Experimental lesion of the foamy type associated with diffusion of eosinophilic material into the adjacent intercapillary space (arrow). Hematoxylin and eosin stain. × 835.



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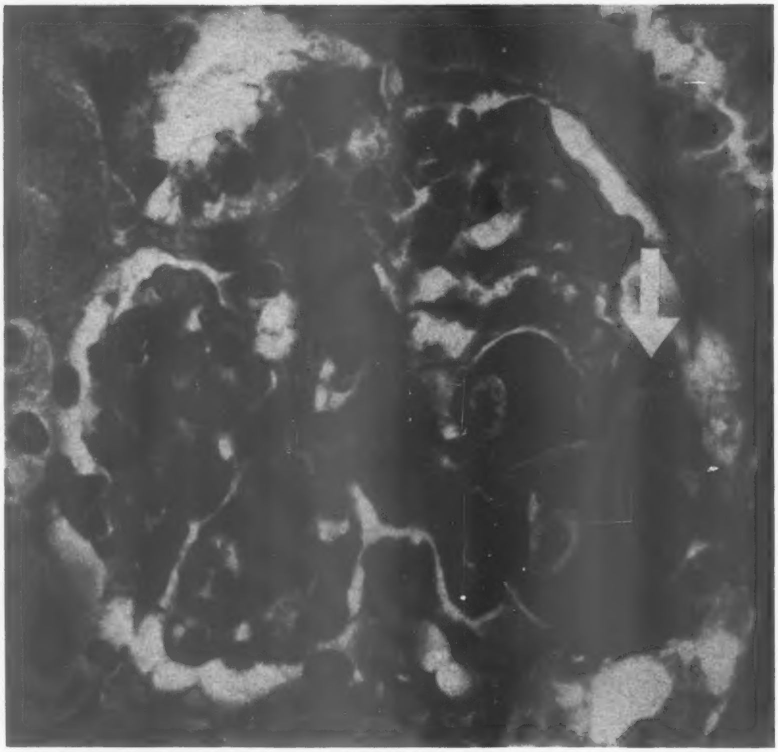
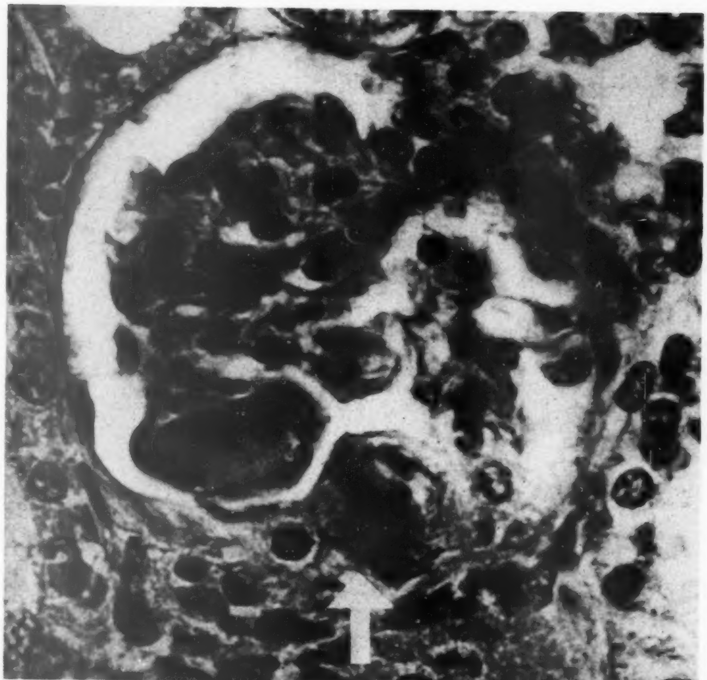


FIG. 9. Rabbit. Experimental lesion of the hyaline type associated with the diffusion of eosinophilic material into the adjacent intercapillary space (arrow). Hematoxylin and eosin stain. × 925.

FIG. 10. Rabbit. "Capsular drop" (eosinophilic material deposited between cells and basement membrane of the parietal layer of Bowman's capsule) (arrow). There is an experimental lesion in the adjacent glomerular tuft. Hematoxylin and eosin stain. × 650.

FIG. 11. Rabbit. Experimental lesion. Silver stain. × 675.



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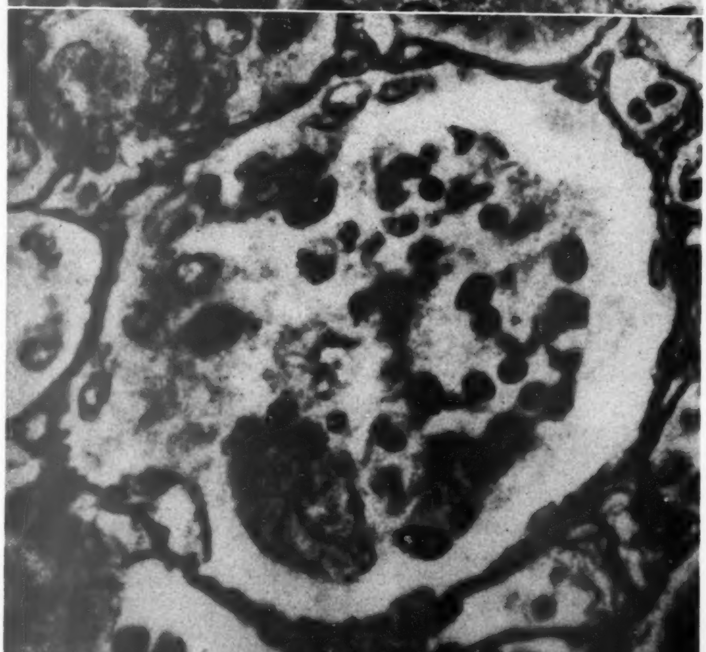

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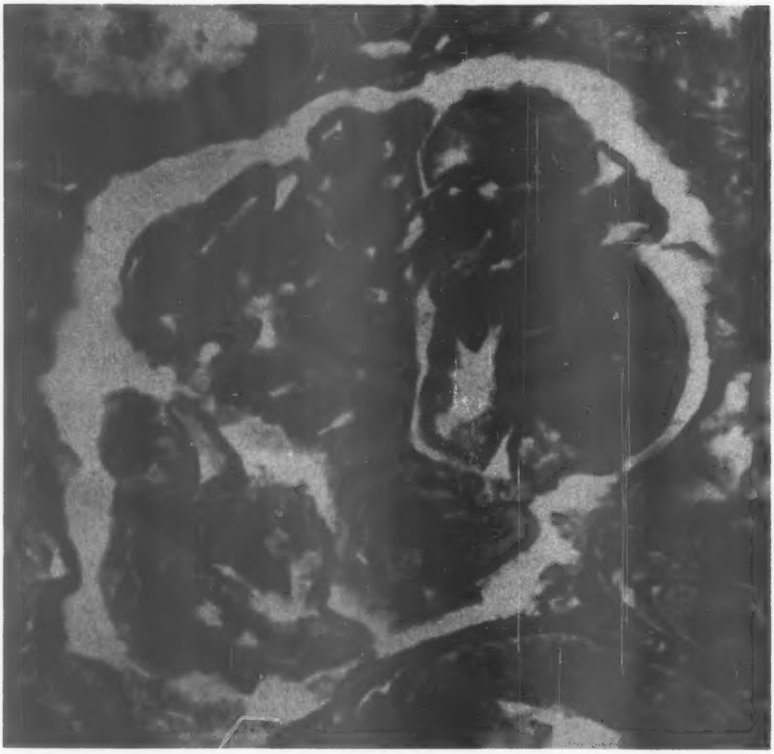
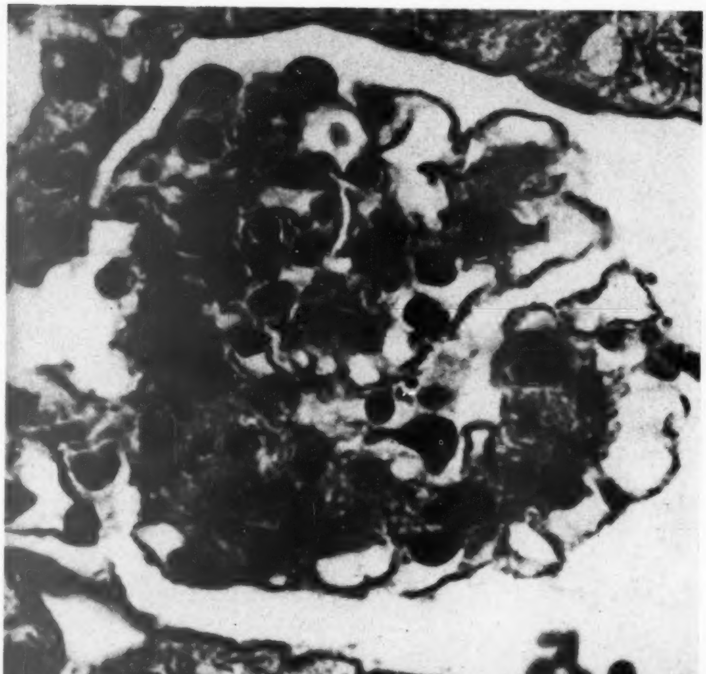


FIG. 12. Rabbit. Experimental lesion, periodic acid-Schiff stain. The experimental lesion stains with the same intensity as the basement membranes. × 1090.

FIG. 13. Rabbit. Long-term study showing extensive intercapillary thickening with patent peripheral capillaries. For comparison with Figure 1. Hematoxylin and eosin stain. × 1215.

FIG. 14. Rabbit. Long-term study showing diffuse intercapillary thickening and beginning nodule formation. For comparison with Figure 1. Hematoxylin and eosin stain. × 835.



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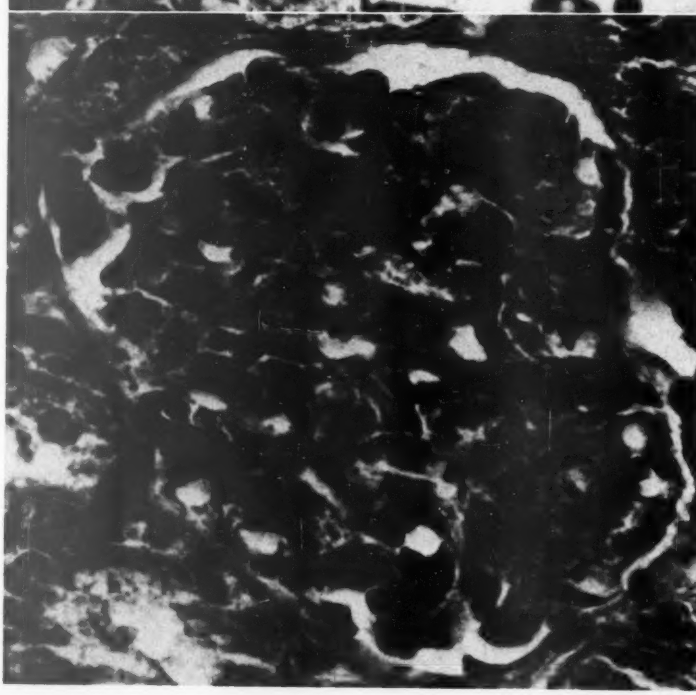

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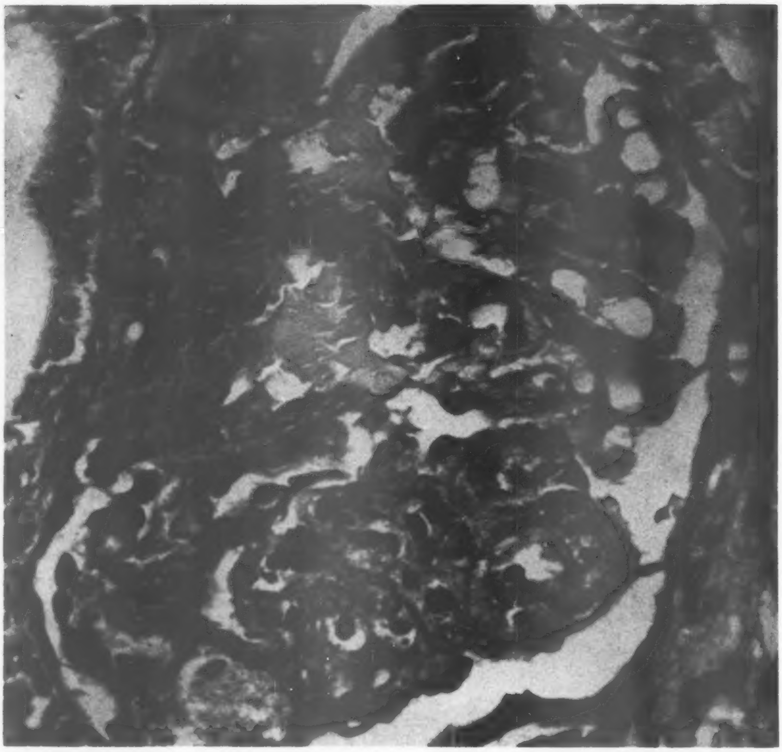
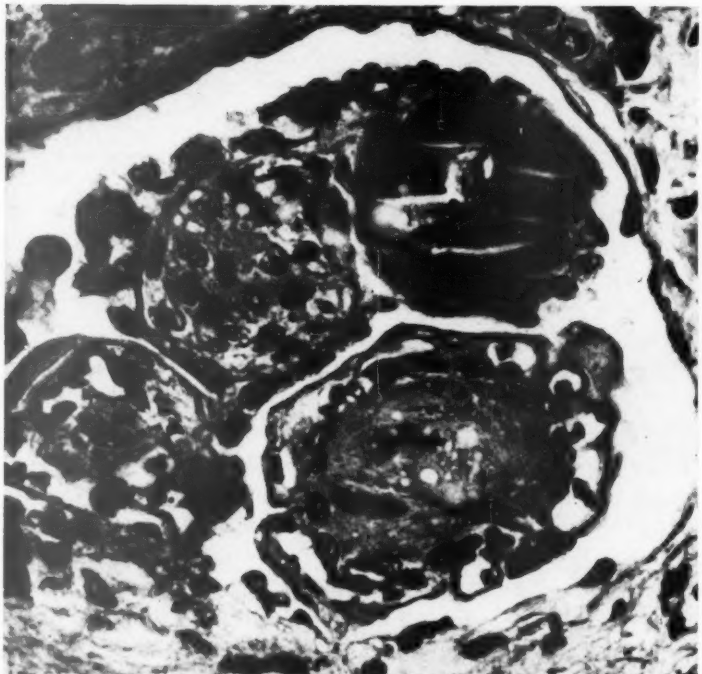


FIG. 15. Rabbit. Long-term study, periodic acid-Schiff-glycerin hematoxylin stain showing that the diffuse thickening is composed of many fine PAS-positive fibrils separating the capillary basement membranes. × 660.

FIG. 16. Human. Glomerulus from patient with diabetic glomerulosclerosis showing two typical nodular lesions with patent peripheral capillaries and one "exudative lesion" of the hyaline type. Hematoxylin and eosin stain. × 595.

FIG. 17. Human. Portion of a glomerulus from a patient with diabetic glomerulosclerosis showing three variations of the "exudative lesion": the hyaline type, foamy type, and vacuolated type. Phosphotungstic acid-hematoxylin stain. × 1080.



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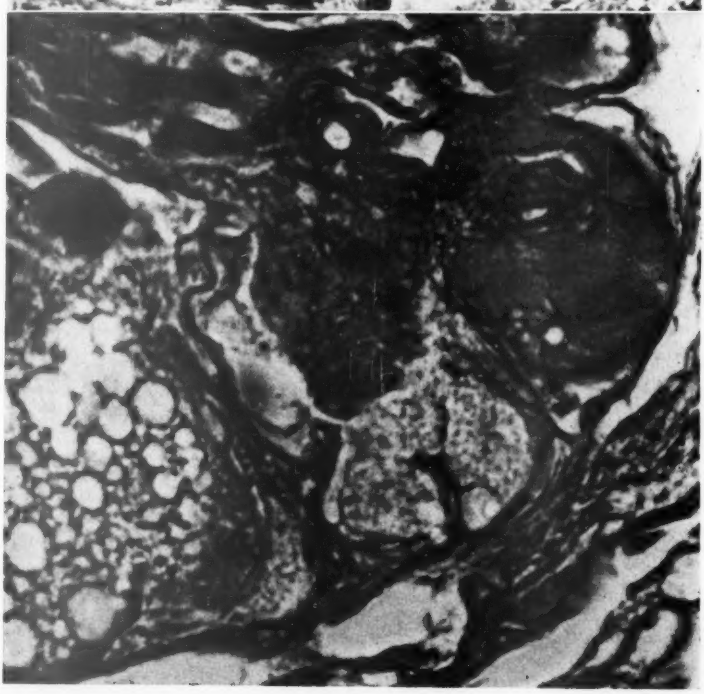

17

PATHOLOGIC EFFECTS OF ANTIMETABOLITES

I. Acute Lesions in the Hypothalamus, Peripheral Ganglia, and Adrenal Medulla Caused by 3-Acetyl Pyridine and Prevented by Nicotinamide *

Samuel P. Hicks, M.D.

From the Departments of Pathology of the New England Deaconess Hospital and Harvard Medical School, Boston, Mass.

The administration of chemical and other agents that act as metabolic inhibitors to adult and developing animals has recently become a useful experimental tool for relating pathologic lesions to the altered metabolic processes that cause them. A number of patterns of injury that simulate natural disease processes have been induced, and they range from acute demyelinization and selective necrosis of cardiac or skeletal muscle to specific injuries in the embryo leading to predictable malformations.[1,2] The increasing interest in antimetabolites in several fields of chemotherapy and nutrition has enhanced the importance of this approach to the nature of specific tissue injury and has led to a systematic investigation of what pathologic changes are induced by these and other metabolic inhibitors. A theoretically ideal approach to characterizing the specific vulnerabilities to disease of the various tissues of the body would be to prepare a series of reagents that would specifically inhibit *in vitro* each step of the known metabolic pathways. These would be administered singly or in combination to animals so that the cells most vulnerable would be damaged and the others spared. Susceptibility to such artificially induced disease patterns might then be catalogued in terms of "enzyme bottlenecks": cell type X utilizing predominantly X metabolic paths would be vulnerable to agents affecting the corresponding enzymes, and cell Y would show a different group of susceptibilities, because of its dependence on a different set of pathways, Y.

There are two principal reasons why such an approach can never be achieved entirely. One is that it is impossible to get all drugs or chemicals in equal concentration into every cell in the body at the same time to measure their different responses. The other is that certain basic metabolic machinery is probably common to all mammalian cells and hence many inhibitors, even though they are highly specific

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in vitro, tend to blanket the metabolism of many types of cells *in vivo*. Experimentally, a near approach to interrupting certain phases of metabolism acutely in all cells of the body at once probably can be had only by such measures as applying radiant energy to the whole body, or depriving it of oxygen, or inducing hypoglycemia with insulin. Here the interruption of metabolic processes in the cells is potentially equal for all cells and the differences in response from one tissue to another are a measure of differences of their dependence on the interrupted pathways. Cerebral cortical and striatal neurons are the cells most vulnerable to deprivation of glucose or oxygen, and from biochemical, physiologic, and pathologic considerations this seems to be related to their obligatory high rate of aerobic oxidation of glucose and lack of stored energy reserves. Primitive differentiating cells in the embryo are the most sensitive of all mammalian cells to acute destruction by ionizing radiations, presumably because some phase of their metabolism, directed primarily to rapid nucleic acid and protein synthesis, is remarkably dependent on enzymes with easily oxidizable prosthetic groups such as sulfhydryl links. Conversely, the adult neuron is relatively radioresistant and the embryonic cell can withstand severe anoxia.

When chemical agents and drugs are introduced into the animal body a number of factors, most of them poorly understood at present, may come into play to alter the even distribution and equal duration of action in all cells. Cell membranes may differ in their permeability to the same and different compounds. The compound may be detoxified at different rates in different tissues, or it may be retained within the body as a whole for a time because of slow excretion. Even when an inhibitor gets into a cell its pathologic effect may be vitiated because the cell has relatively large amounts (activity) of the enzyme, or alternate metabolic pathways may offer an escape for the cell. Assuming, however, that a certain compound, sodium cyanide for example, may be rapidly distributed by the blood to virtually every tissue in the body, still another important consideration arises. The blood supply of one region may deliver the compound more rapidly and also clear it more rapidly than that of another region. Blood flow could in this way modify duration of action, even though other factors might be nearly equal.

Despite these obstacles to interpretation of the pathologic effects of metabolic inhibitors, a number of patterns of experimental injury have emerged which seem to indicate that the vulnerability to injury is more closely related to enzyme inhibition than any other single factor.

In adult animals the oligodendroglia-myelin sheath complex of white matter can be selectively damaged by cyanide, azide, and malononitrile, the common action of which is presumed to be the inhibition of cytochrome oxidase, an enzyme found in low concentration in white matter. Blood flow may be an enhancing factor here, as has been noted, for these compounds damage the cortical gray matter simultaneously, as would be expected. Nevertheless, when these three compounds, chemically and physiologically quite different, produce the same lesion, the cytochrome oxidase enzyme bottleneck for this tissue complex seems to be a prime factor. Similarly, primitive differentiating embryonal cells are vulnerable to several radiomimetic drugs and sulfhydryl reagents, which differ in virtually every way except their common effects *in vitro* on sulfhydryl groups.

The administration of analogues of several vitamins and amino acids to adult and developing animals has produced still other patterns of injury, and these can be prevented by the simultaneous or prior administration of the corresponding normal metabolite. The first of these, acetyl pyridine, is a close chemical analogue of nicotinamide. It has produced consistently the pattern of injury summarized in the title of this report, and the lesions may be completely prevented by nicotinamide. It is the twofold purpose of this paper, first, to report these findings and, second, to consider, in the light of past experiments, the fundamental validity of the use of antimetabolites in certain studies of metabolism.

Methods

The general plan of approach in investigations of this type has been to administer a sublethal dose (often about the LD_{50} dose) of the agent under study to a series of male and female adult animals, pregnant animals bearing fetuses, and newborn animals. Rats and mice are used principally and they are prepared for morphologic study usually about 24 hours after treatment, when enough time has elapsed for those tissues, which will be selectively effected, to show irreversible lesions, that is, necrosis of vulnerable cells. Sometimes, as with acetyl pyridine, plasmocid,[1,2] and ionizing radiations, actual necrosis of affected cells is clearly evident microscopically in the unusually short time of a few hours after administration. More often, as is generally observed in acute destructive processes, a longer period is required before unequivocal evidence of selective necrosis develops. Whether the agent has to be administered in something approaching the LD_{50} dose or considerably less depends entirely on the "margin of safety" between the agent's specific effects on certain tissues and its general

lethal effect on the whole animal. Since the whole aim of this series of studies has been to search out *differences* among cells and tissues, it goes without saying that toxic agents that seem to have a blanketing effect on many phases of metabolism and after the administration of which animals either die without selective lesions or recover completely, have not received detailed attention here.

Usually the experimental animal was subjected to a complete gross and microscopic necropsy, but in some cases in which a pattern of lesions seemed well established, additional experiments were carried out with necropsies limited to specific tissues of interest, or more extensive samples were made of one tissue, such as muscle or skeleton. The complete necropsy included examination of: brain (in serial sections when indicated); spinal cord and associated nerves and ganglia at two or three levels; eyes; trigeminal ganglia and sciatic nerve; muscles of the larynx, pharynx, jaw, tongue, intercostal region, diaphragm, abdominal wall and spine; heart; great vessels; lungs; thymus; lymph nodes; and neck organs including thyroid, salivary, and often parathyroid glands; liver; spleen; pancreas; stomach; esophagus; large and small intestine; kidney; bladder; prostate; seminal vesicles; testis; ovary and tube; adrenal glands; vertebrae; ribs; base of skull; skin; and adipose tissues. Sections of the internal ear and related mastoid bone were examined from some animals. The parathyroid gland did not appear in every section of neck organs and for technical reasons the pituitary body occasionally was lost. Breast tissue has not been studied in this series.

In the acetyl pyridine experiments, 27 adult animals were given the drug and subjected to complete necropsy as outlined. An effective dose range had been established and 11 mg. (0.01 cc.) was fatal within a few hours. All animals showed lesions (see Results). Three were rats (Wistar albino strain) and 24 were Swiss albino, ABC and BAF_2 mice of both sexes. Five mice were pregnant, each with 5 to 7 fetuses which also were examined. One newborn mouse was studied (sole survivor of 5 treated with acetyl pyridine). Necropsies were performed 4 to 24 hours after treatment. Adult mice weighing 25 to 35 gm. received 4 to 11 mg. of acetyl pyridine in aqueous solution or Wesson vegetable oil suspension, or in pure form either subcutaneously or peritoneally. Acetyl pyridine is a liquid, 1 cc. of which weighs about 1.1 gm. (uncorrected).

Additional experiments were carried out in mice to determine whether the lesions produced by acetyl pyridine could be prevented by the simultaneous administration of nicotinamide. Eight Swiss al-

bino male and female mice were given 10 mg. of nicotinamide (Abbott ampoules, 100 mg. in 2 cc. of H_2O) *intraperitoneally* and within 5 minutes approximately 11 mg. (0.01 cc.) of acetyl pyridine *subcutaneously*. Eight mice were given the acetyl pyridine without nicotinamide at the same time. The mice protected with nicotinamide survived without lesions; the unprotected ones died within 16 hours. Complete necropsies and serial sections were done on the brains of the animals receiving both nicotinamide and acetyl pyridine. The serial sections were done to obtain conclusive evidence of protection by the nicotinamide. A few experiments on still other mice showed that 2 to 5 mg. of nicotinamide did not protect against 11 mg. of acetyl pyridine.

Additional series of experiments comparable to those with acetyl pyridine were carried out with sublethal doses of pyridine-3-sulfonic acid and alpha picolinic acid, both of which often are listed as analogues of nicotinic acid. Also, mice were kept on a nicotinic-acid-free diet from 3 to 6 weeks but were not prevented from eating their feces. These experiments need not be detailed further, but it is appropriate to mention them for the discussion which follows.

Results

All 24 adult mice and 3 rats treated with acetyl pyridine showed the pattern of destructive lesions, to be described. The newborn and fetal mice showed only lesions of peripheral ganglion cells. The more common findings in adults were necrosis of the adrenal medulla, and of neurons of the spinal and sympathetic ganglia of the supra-optic nucleus of the hypothalamus and of the pyramidal layer of the hippocampus. Ganglion cell necrosis occurred in all animals; adrenal lesions in all but one (4-hour animal). Supra-optic nucleus damage was almost always seen as was hippocampal pyramidal cell necrosis. Less frequently there was necrosis of neurons in the medial amygdaloid nuclei, fascia dentata neurons of the hippocampus, and neurons situated ventrally and laterally in the medulla. The latter were not limited to specific nuclei but did involve fairly often the inferior olives and some cells in the fifth and seventh nerve nuclei. The cerebral cortex and dorsal striatum, so markedly affected by other factors such as anoxia, hypoglycemia, and the cyanide group, escaped damage. A few large neurons in the ventral anterior striatum just dorsal to the amygdaloid nucleus were damaged in 2 animals and in 2 animals a few large neurons of the substantia nigra were necrotic. In one animal a few neurons of the habenular and the reuniens nuclei also were damaged. In only one of the 27 animals were a few necrotic

trigeminal ganglion cells seen. Thus a marked difference exists between trigeminal and other peripheral ganglia. Plasmocid[1,2] caused selective necrosis of trigeminal neurons and cardiac and skeletal muscle, but never damaged the spinal or sympathetic ganglion cells.

The 8 animals given nicotinamide with a lethal dose of acetyl pyridine showed none of these lesions. In these animals the brains were serially sectioned, and complete necropsies made. The only positive finding was dilation of the lateral cerebral ventricles, a finding noted also in animals that received large doses of nicotinamide alone.

Fetuses from 5 pregnant mice treated with acetyl pyridine were studied by means of several whole body sections from each. Necrosis of spinal ganglion cells, ranging from a few to virtually all cells, was seen in all fetuses. One newborn mouse, the survivor of a group of 5 given acetyl pyridine, showed necrosis of spinal ganglion cells. In some of the fetuses, necrosis of cochlear and trigeminal neurons occurred, but the adrenal medulla and other parts of the brain and viscera were unaffected except for scattered necrotic neurons of the brain stem.

The criteria for recognizing pathologic changes have been detailed in earlier studies. In the adult nervous system unequivocally necrotic neurons develop an eosinophilic cytoplasm, sometimes poorly outlined and markedly reduced in volume, within 5 to 20 hours. The neuron nucleus also loses volume, becomes strongly basophilic and solid, but poorly outlined, and then may fade. In the medulla of the adrenal gland similar changes occur, but eosinophilia is not conspicuous. After acetyl pyridine and plasmocid, necrosis of ganglion cells could be seen in a few hours. In previous experiments with cyanide and anoxia, at least 6 hours was necessary before unquestionable irreversible damage could be recognized, and 12 to 18 hours usually was allowed to elapse before study.

The fact that the drug was given intraperitoneally in some instances and subcutaneously in others, and undiluted or in aqueous solution or in oily suspensions, had no evident influence on the lesions. The larger the dose the more extensive the lesions were in the sites described. Animals that received 0.01 cc. of pure acetyl pyridine were studied 5 hours after administration of the drug. They would have died at this time and were moribund when killed for necropsy. All other animals had fully developed lesions when studied the day after the drug was given.

As to the functional changes associated with acetyl pyridine administration, it is notable that, in contrast to a number of other chemicals

that produce brain lesions, this compound did not often produce convulsions. Except with large doses after which death was accompanied by general clonic convulsions, the animals usually became increasingly quiet, weak, and then moribund. In the hours before spontaneous death or sacrifice for necropsy they took little or no food or water. They seemed to be especially weak in the hind limbs. Their bladders became distended with urine; in fact, this prevented the measurement of urinary output in several additional mice to learn whether diabetes insipidus was associated with the lesions of the supra-optic nucleus. The animals did not appear to be in circulatory shock or to suffer respiratory embarrassment. The drug produced its pathologic changes rapidly and within a few hours, but death usually did not result before 24 hours with effective doses below 11 mg.

The experiments with alpha picolinic acid (10 mice) and pyridine sulfonic acid (30 mice) may be summarized by saying that the former produced necrosis of neurons in the mid brain colliculi and inferior olivary nuclei of some animals, whereas the latter caused hippocampal necrosis in but a single animal. Nicotinamide given simultaneously with picolinic acid prevented neither the lesions nor death.

Mice kept on the nicotinic-acid-free diet showed no lesions comparable to those caused by acetyl pyridine.

Examples of the lesions caused by acetyl pyridine are shown in Figures 1 and 2.

Discussion

Acetyl pyridine produces a distinctive acute pattern of injury in the neuraxis and adrenal glands in mice and rats and this is prevented by the simultaneous administration of nicotinamide. There is no reason to doubt that this is a competitive reaction between metabolite and antimetabolite, but what is its relation to nicotinamide deficiency in particular and brain metabolism in general? Is it valid to assume that the administration of an antimetabolite to a living animal is indeed analogous to producing the corresponding deficiency even when the results are supported by morphologic studies? There is no naturally occurring acute pathologic syndrome comparable to the pattern of injury produced by acetyl pyridine, and thus far it has not been possible to imitate the pattern of chronic nicotinamide deficiency in adults of these species.

Two conclusions regarding the acetyl pyridine experiments are possible:

(1) The lesions represent an accelerated picture of nicotinamide deficiency and the cells involved are specifically more dependent on

pyridine nucleotide enzymes, of which nicotinamide is a prosthetic group, than other cells.

(2) Acetyl pyridine selectively affects certain cells by reason of peculiarities of blood supply, cell permeability, detoxification rates, or other factors noted in the introductory paragraphs, and its effects are therefore essentially the result of caprice.

In searching for the mechanism of action of acetyl pyridine, possible common anatomical features or physiologic interrelationships of the cells involved need to be explored. The blood supply varies for each of the involved regions. The supra-optic and paraventricular nuclei possess some of the richer capillary beds of the nervous system and apparently a rapid blood flow, while the posterior root and sympathetic ganglia have one of the poorer; the hippocampus lies somewhere between, and the adrenal medulla has a sinusoidal bed. Thus a type of blood supply common to all regions, which might cause a slow clearance of acetyl pyridine once delivered to the region, cannot be established.

Regarding differences in permeability, the hypothalamic neurons and adrenal medulla cells have the most intimate relation of cell to capillary, but this does not hold for the others. Information on the actual cell membrane for each variety of cell is, of course, lacking and not presently susceptible to direct measurements *in situ*. Acetyl pyridine, pyridine sulfonic acid (as neutral sodium sulfonate), and nicotinamide are pyridine derivatives with their side groups in the beta position.[8] Alpha picolinic acid (pyridine alpha carboxylic acid) is an isomer of nicotinic acid (pyridine beta carboxylic acid). Acetyl pyridine seems closest to nicotinamide chemically and however the experiments may be interpreted, the two compounds must be able to move about in the body and in and out of cells and their enzyme systems with remarkable similarity.

Light may be thrown on the problem by considering some observations made on the changing metabolism of the nervous system during development. It is known[1,2] that the newborn rat and mouse can withstand prolonged anoxia and that this is related largely to the capacity of the inter-brain, brain stem, and cord to utilize glucose anaerobically. Such animals live virtually unharmed in nitrogen for 40 to 55 minutes and, when removed at the end of this period, they have sustained no lesions. Glycogen stores are used up during the anoxic period. Fluoride, insulin, and especially iodoacetate will shorten this anaerobic survival in nitrogen, but with these drugs the animals can live in air. The action is apparently to interrupt the Embden-Meyerhof path of

glycolysis,[4] or, in the case of insulin, to restrain the mobilization of glycogen stores. The brain stem and cord seem to be the vulnerable regions but the heart also may be an important target of this combined metabolic insult. In air the aerobic stepwise breakdown of glucose by the Warburg-Dickens scheme may be utilized, as is presumed to be the obligate method in the adult brain. Acetyl pyridine also has been shown to shorten anaerobic survival substantially in experiments comparable to those with iodoacetate, which suggests that pyridine nucleotide enzymes may be especially important to the anaerobic glycolytic paths. Both developmental changes related to metabolism and physiologic observations on the relative resistance to anoxia of the brain stem and cord indicate that these regions have a different metabolism from the forebrain. The evidence suggests that these regions may carry over some of the dependence on the Embden-Meyerhof path into adult life and that it may serve at the same time to provide these lower levels of the neuraxis with some capacity for anaerobic survival as contrasted with cerebral cortex and anterior striatum.

Altogether these data would tend to support the first conclusion given, that the action of acetyl pyridine is much more a reflection of a characteristic pattern of metabolism of certain parts of the neuraxis than simply a result of caprice.

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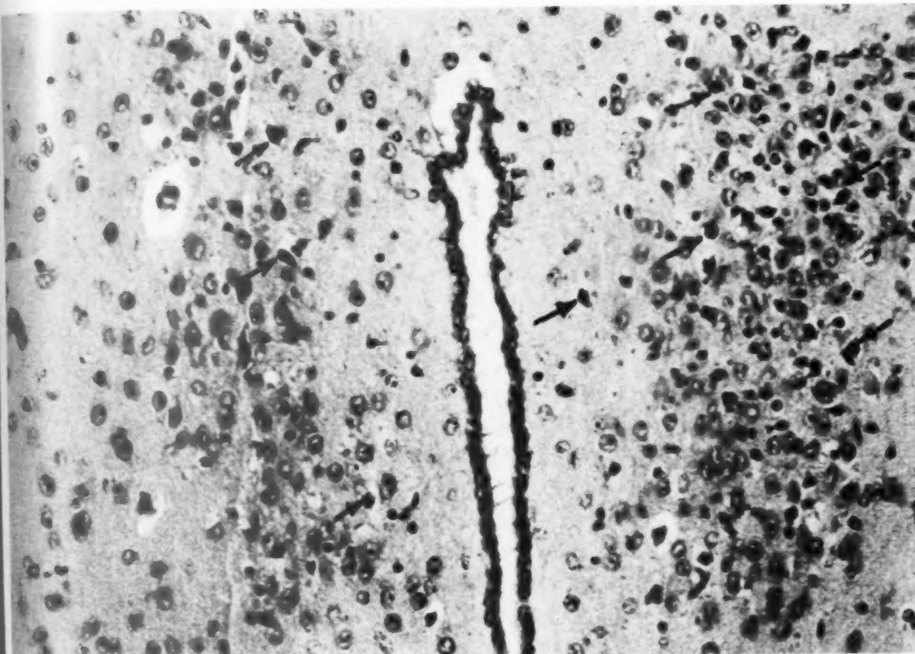
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Acute necrosis of neurons of the paraventricular nucleus of the hypothalamus of a mouse, caused by acetyl pyridine. Hematoxylin and eosin stain. × 400.

FIG. 2. Acute necrosis of spinal ganglion neurons in a mouse, caused by acetyl pyridine. Hematoxylin and eosin stain. × 750.



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